THE

AUSTRALIAN JOURNAL OF EXPERIMENTAL BIOLOGY AND MEDICAL SCIENCE

Sustained since 1926 by the Endowment of Sir Joseph Cooke Verco for the publication of Medical Research

EDITOR-IN-CHIEF:

MARK L. MITCHELL (ADELAIDE)

CO-EDITORS:

J. B. CLELAND (ADELAIDE), SIR STANTON HICKS (ADELAIDE)

Assisted in the selection of papers for publication by the following

BOARD OF EDITORS:

A. A. ABBIE (Adelaide); L. B. BULL (Melbourne); SIR MACFARLANE BURNET (Melbourne); F. S. COTTON (Sydney); H. F. HOLDEN (Melbourne); E. V. KEOGH (Melbourne); R. LEMBERG (Sydney); I. M. MACKERRAS (Brisbane); H. R. MARSTON (Adelaide); J. A. PRESCOTT (Adelaide); W. RAY (Adelaide); C. W. STUMP (Sydney); E. R. TRETHEWIE (Melbourne); V. M. TRIKOJUS (Melbourne); J. S. TURNER (Melbourne); E. J. UNDERWOOD (Perth); H. J. WILKINSON (Brisbane); J. G. WOOD (Adelaide); R. DOUGLAS WRIGHT (Melbourne).

VOL. XXIX

PUBLISHED BY THE UNIVERSITY OF ADELAIDE

MANAGED FOR THE UNIVERSITY BY THE MEDICAL SCIENCES CLUB OF SOUTH AUSTRALIA

PRINTED AT THE HASSELL PRESS, ADELAIDE

1951

JUN 16 52 U of M Bindery

B570.5
Au78

CONTENTS

PART 1. JANUARY, 1951

PAGE.

STEPHEN FISHER: "Antigenic relationships of some erythrocyte adsorbable fractions of some Mycobacteria" 1

E. C. POULKES: "The effect of atebrin on reduction of haemiglobin (methaemoglobin) in rat erythrocytes" 9

G. F. HUMPHREY AND H. L. WEBSTER: "The deaminase activity of myosin" 17

E. L. FRENCH, G. S. GUNTER AND R. MOTTERAM: "Ovarian cyst contents as a source of virus inhibitor: histological, chemical, and rheological studies" 23

K. W. CLELAND: "The enzymatic architecture of the unfertilized oyster egg" 35

HENRY FRANCIS HOLDEN AND JEAN SCOTT: "A simple apparatus for drying small specimens of sera" 47

LILA HAYES: "Specific serum agglutination of sheep erythrocytes sensitized with bacterial polysaccharides" 51

P. HOLMES: "The arginine content of certain seed proteins" 63

E. R. TRETHEWIE AND FAY M. GAFFNEY: "The presence of 'bound' histamine following the injection of histamine acid-phosphate into the perfused liver" 67

PART 2. MARCH, 1951

VERA I. KRIEGER AND T. B. KILVINGTON: "The failure to detect anti-diuretic substance in the blood and urine of dehydrated rats" 77

JOAN B. CLELAND: "Cystine, methionine and sulphate excretion in cystinuria" 85

D. I. ANNEAR: "Mixed bacterial growth. III. Studies on the mixed growths of a viridans streptococcus, a diphtheroid and *Staphylococcus albus*" 93

G. REID: "The pharmacology of tryptamine" 101

PHILIP A. TRUDINGER: "Deamination of aspartic acid by *Proteus* X-19. I. Stimulation of anaerobic deamination by glucose, adenosine and glutathione" 117

L. R. FINCH AND E. J. UNDERWOOD: "The influence of clover leys on the thiamin and nitrogen contents of wheat" 131

MARGARET DONNELLEY: "Studies in experimental immunology of influenza. VII. An improved complement-fixation technique" 137

D. I. ANNEAR: "The inoculation, cultivation and enumeration of bacteria on glass surfaces. Methods for disinfection studies" 147

F. H. SHAW, MONICA MACCALLUM, D. J. DEWHURST AND J. F. MAINLAND: "The possibility of the dual nature of sympathetic ganglion cells. III" 153

PART 3. MAY, 1951

GEORGE READ: "Adrenal transplants into spleen and the inactivation of adrenal cortical hormones" 161

ALICK ISAACS AND MARGARET EDNEY: "Interference between inactive and active influenza viruses in the chick embryo. V. The behaviour of different strains of challenge virus" 169

ALICK ISAACS AND MARGARET EDNEY: "Interference between inactive and active influenza viruses in the chick embryo. VI. The behaviour of different strains of interfering virus" .. 179

MARGARET GEDDES AND G. F. HUMPHREY: "Glycolysis in *Paramecium caudatum*" 187

J. J. LAWRENCE: "The growth of leptospirae in semi-solid media" 195

F. C. COURTICE, W. J. SIMMONDS AND A. W. STEINBECK: "Some investigations on lymph from a thoracic duct fistula in man" 201

H. HOFFMAN: "Fate of interrupted nerve-fibres regenerating into partially denervated muscles" .. 211

SIBELY J. McLEAN: "The isolation and characteristics of bacteriophages from staphylococci of animal origin" 221

F. E. BINET, E. R. TRETHEWIE AND FAY M. GAFFNEY: "The extraction of liver anticoagulant other than heparin" 233

G. REID: "Adenosine triphosphate and the adrenalin-liberating action of serum" 243

1385673

PART 4. JULY, 1951

Page

H. W. BENNETTS AND E. J. UNDERWOOD: "The oestrogenic effects of subterranean clover (*Trifolium subterraneum*). Uterine maintenance in the ovariectomised ewe on clover grazing" 249

F. C. COURTICE AND W. J. SIMMONDS: "The removal of protein from the subarachnoid space" 255

M. F. WARBURTON AND S. FISHER: "The haemagglutinin of *Haemophilus pertussis*. III. Extraction of the antigen from the bacteria and its stabilization by adsorption" 265

A. B. BECK AND A. W. BRADEN: "Studies on the oestrogenic substance in subterranean clover: (*Trifolium subterraneum L.* var. Dwalganup)" 273

R. D. WRIGHT AND V. WYNN: "Digestion of protein in the alimentary canal" 281

K. T. H. FARRER: "The liberation of pantothenic acid from autolysing yeast cells" 285

H. HOFFMAN: "A study of the factors influencing innervation of muscles by implanted nerves" .. 289

NEIL D. CROSBY AND DAVID I. B. KERR: "Comparative effects of the nicotine alkaloids upon respiration" 309

PART 5. SEPTEMBER, 1951

E. R. TRETHEWIE AND FAY M. GAFFNEY: " The influence of diphtheria toxoid on the histamine content of the lung and the lethal dose of histamine" 315

H. HARRIS: "The preformed ammonia content of skeletal muscle and liver in experimental anuria" 321

S. FAZEKAS DE ST.GROTH, M. DONNELLEY AND D. M. GRAHAM: "Studies in experimental immunology of influenza. VIII. Pathotopic adjuvants" 323

S. FAZEKAS DE ST.GROTH: "Studies in experimental immunology of influenza. IX. The mode of action of pathotopic adjuvants" 339

F. M. BURNET AND MARGARET EDNEY: "Recombinant viruses obtained from double infections with the influenza A viruses MEL and Neuro-WS" 353

NEVILLE F. STANLEY: "Purification of Australian strains of Coxsackie virus" 363

NEVILLE F. STANLEY, LILA HAYES AND D. C. DORMAN: "Suckling mouse viruses in New South Wales; their relationship to the Coxsackie group of viruses" 367

R. N. LYONS: "Thrombin components in blood coagulation" 383

J. S. ROBERTSON: "Some developments in rabbit ear chamber techniques" 393

PART 6. NOVEMBER, 1951

G. REID AND M. RAND: "Physiological actions of the partially purified serum vasoconstrictor (Serotonin)" 401

H. HOFFMAN AND P. H. SPRINGELL: "An attempt at the chemical identification of 'Neurocletin' (the substance evoking axon-sprouting)" 417

S. FISHER, M. F. WARBURTON, H. N. B. WETTENHALL AND S. W. WILLIAMS: "The haemagglutinin of *Haemophilus pertussis*. IV. Reactions and antibody response in babies and children following the administration of haemagglutinin adsorbed on aluminum phosphate" .. 425

P. FANTL, J. F. NELSON AND G. J. LINCOLN: "The absorption and metabolism of substances with vitamin K activity under normal conditions and after biliary obstruction. ..A study in man and rabbits, including a vitamin K saturation test" 433

E. R. TRETHEWIE: "The influence of sodium salicylate and acetyl salicylic acid on the release of histamine in anaphylaxis" 443

F. C. COURTICE AND A. W. STEINBECK: "The effects of lymphatic obstruction and of posture on the absorption of protein from the peritoneal cavity" 451

A. T. DICK AND J. B BINGLEY: "Further observations on the determination of molybdenum in plant and animal tissue" 459

N. ATKINSON, J. DINEEN AND J. S. ROBERTSON: "A Pleurodynia virus pathogenic for infant mice" 463

S. ROSE: "The role of the adrenal cortex in diabetic ketosis" 469

ANTIGENIC RELATIONSHIPS OF ERYTHROCYTE ADSORBABLE FRACTIONS OF SOME MYCOBACTERIA

by STEPHEN FISHER

(From the Commonwealth Serum Laboratories, Melbourne).

(Accepted for publication 2nd October, 1950.)

Middlebrook and Dubos (1948) found that extracts of the phenol-insoluble fraction of tubercle bacilli adsorbed firmly on washed sheep erythrocytes and sensitized them to agglutination by specific antisera. Haemolysis of the sensitized red cells in the simultaneous presence of antiserum and complement was reported from this laboratory (Fisher and Keogh, 1950; Fisher, 1950).

In the course of work on the sera of bovines, some anomalous reactions were encountered which led to an investigation into the specificity of the reaction. The results reported in this paper show that sera of animals inoculated with various species of Mycobacteria, and sera of human subjects suffering from leprosy agglutinate and, in the presence of complement, lyse, erythrocytes sensitized with extracts of the phenol-insoluble fraction of tubercle bacilli. A full study of the cross-reactions between antisera, and homologous and heterologous extracts was therefore undertaken.

Materials and Methods.

Strains. Organisms of the following strains, grown on synthetic medium, killed by heat, washed and dried, were obtained from the Tuberculin Department: *Myco. tuberculosis*, human type (strain PN, virulent), bovine type (strain CH 29), murine type (strain OV 164) and avian type (strain VS); and Johne's bacillus (strain TEPS). A strain of *Myco. phlei* was subcultured from nutrient agar slopes into Hartley's broth or Ward's tryptic digest broth and grown at 37° C. for a few days; the organisms were then collected on the centrifuge, washed once with saline, steamed for one hour and dried.

Suspensions of the dried organisms were used for the immunization of rabbits and for the preparation of extracts. Organisms of strain CH 29 (bovine type of tubercle bacillus) were found unsatisfactory for the latter purpose and were replaced by semi-dry BCG "cheese" obtained from the BCG Department of these Laboratories.

Preparation of extracts. The dried organisms were extracted with 90 p.c. phenol as described in the previous paper (Fisher, 1950). Since the BCG "cheese" contained 50–60 p.c. water, the phenol extraction of this material was commenced by grinding it up with 5 times its weight of phenol crystals; the extraction was then continued in the usual way. The phenol-insoluble residues were extracted with the methanol-phosphate-saline used by Middlebrook and Dubos (1948), as described before (Fisher, 1950). The extracts were stored in the cold room for periods up to 3 months without apparent loss of potency.

Immunization of rabbits. Suspensions containing 2 mg. of the dried organisms per ml. were prepared in 0·2 p.c. formalin saline by grinding. Two rabbits were immunized with each suspension. Eight bi-weekly intravenous injections of 1 ml. suspension were given, followed by a ninth dose a week later. Ten days after the last dose the rabbits were bled from the heart. The sera of the animals receiving the same suspension were pooled, inactivated, Seitz filtered and preserved in the cold room with 0·01 p.c. merthiolate.

Sera from two rabbits, immunized with strains of *Myco. ulcerans* (MacCallum, Tolhurst, Buckle and Sissons, 1948) were obtained from Prof. F. J. Fenner.

Sera from immunized bovines were obtained from Mr. T. S. Gregory. Young animals from the healthy stock of the C.S.I.R.O. Experimental Farm at Tooradin, which had failed to react to intradermally injected tuberculin, were used. The 53 animals were divided into five groups, four of which were immunized with tubercle bacilli of the human, bovine and avian types, and with Johne's bacillus, respectively; the immunization consisted of a single injection of 25 mg. heat-killed organisms suspended in 12·5 ml. paraffin and 5 ml. Falba. The animals of the fifth group were not immunized and served as controls. Serum specimens were obtained between the 14th and 16th weeks of the post-immunization period.

Sera from cases of leprosy. Serum specimens of six aborigines suffering from leprosy and from five aborigines free from the disease were supplied by Dr. L. W. Alderman, from Darwin. No sign of tuberculosis could be detected in these subjects by clinical examination or radiography of the chest.

Sheep erythrocytes were obtained, preserved and washed as described in the previous paper (Fisher, 1950).

Complement. Fresh guinea-pig serum was used.

Haemagglutination and haemolytic tests. Sera and complement were absorbed with normal sheep erythrocytes, red cells were sensitized with the extracts of the phenol-insoluble residues of the bacteria, and haemagglutination and haemolytic titrations were carried out using methods the details of which have already been described (Fisher, 1950) and will not be elaborated on here unless different from those previously employed. Depending on the potency of the sensitizing extracts, between 10 and 50 drops were used to coat one drop of packed red cells.

Titres are expressed in terms of reciprocals of highest serum dilutions giving complete haemagglutination, or haemolysis, respectively.

RESULTS

Agglutination of erythrocytes sensitized with extract of tubercle bacilli of the human type by sera of rabbits immunized with different species of mycobacteria.

The following titres were obtained:

Immunizing agent	Titre
Human TB	400
Bovine TB	80
Murine TB	80
Avian TB	640
Johne's bacillus	320
Myco. phlei	40

All sera thus agglutinated the sensitized cells to significant titres; the highest titre was given by a heterologous antiserum. 1:5 dilutions of the serum specimens obtained from the rabbits prior to immunization all failed to agglutinate cells sensitized with the respective homologous extracts.

Titrations of haemolytic antibody in immune rabbit sera against erythrocytes sensitized with homologous and heterologous extracts.

Erythrocytes were sensitized with extracts of each of the six species of acid-fast organisms. Each suspension was tested against the six antisera in haemolytic titrations. High titres were obtained in all the titrations (Table 1). The titres of the sera from the pre-immunization bleedings, in tests against cells coated with the homologous extracts, in no case exceeded 10.

TABLE 1.

Titres of haemolytic antibody in sera of immunized rabbits against erythrocytes sensitized with homologous and heterologous extracts.

Serum prepared against	Sensitizing extract prepared from					
	Human TB	Bovine TB	Murine TB	Avian TB	Johne's bacillus	*Myco. phlei*
Human TB	6,400	1,280	640	160	640	6,400
Bovine TB	2,560	320	400	200	100	640
Murine TB	1,280	100	320	800	1,600	3,200
Avian TB	10,240	6,400	6,400	640	1,600	3,200
Johne's bacillus	2,560	800	1,600	1,600	640	1,600
Myco. phlei	3,200	800	200	400	800	6,400

The degree of antigenic overlap cannot be assessed from the titres of haemolytic antibody for the following reasons: (i) it was not practicable to carry out the tests at the same time, with the same batch of cells or even, in some cases, with the same batches of sensitizing extracts; (ii) the observed haemolytic titre depends on the degree of sensitization as well as on serum antibody level (Fisher, 1950 and unpublished results).

Haemolytic antibody against erythrocytes coated with extract of tubercle bacilli of the human type in the sera of immunized bovines.

Fifty-three sera were titrated individually. Each batch of tests included a titration of a reference rabbit serum as a positive control. While none of the sera from the five immunized control animals reached a titre of 5, the titres of the sera of the immunized animals varied between 40 and 2,560; the means of the titres in each group are given in Table 2. While the antibody level was significantly higher in any of the immunized groups than in the control group the difference between the mean titres of the immunized groups was not significant.

The above experiments indicate cross-over between the erythrocyte adsorbable antigenic fractions of the six species of Mycobacteria investigated.

TABLE 2.

Titres of haemolytic antibody against erythrocytes sensitized with extract of tubercle bacilli of the human type in sera of immunized bovines.

Immunizing agent	Number of animals in group	Geometrical mean of titres	5 p.c. limits of geometrical mean ($\pm 2\sigma$)
Human TB	15	510	298–865
Bovine TB	17	410	246–676
Avian TB	8	230	90–598
Johne's bacillus	8	320	189–540
Controls (not immunized)	5	<5	

Neutralization of haemolytic antibodies with homologous and heterologous extracts.

In order to estimate the degree of antigenic overlap between the red cell adsorbable fraction of the six strains under investigation, series of tests were set up with the rabbit immune sera. The tests were based on the observation (Fisher, 1950) that addition of the sensitizing extract to the serum is capable of neutralizing the haemolytic antibody. Two strains were compared in each experiment; the 15 possible combinations between pairs of the six strains were tested in turn. The sera were always set up against cells coated with the homologous extract; the addition of the sensitized cells was preceded by admixture to the serum dilutions of graded amounts of homologous or heterologous extracts; complement was added last. Preliminary tests showed that the neutralization patterns obtained by this technique were independent of the absolute amount of antibody in the sera, of minor variations in the sensitizing power of the extracts and of possible variations in the relative sensitizing and antibody fixing potencies of the extracts. The details of the technique of each experiment were as follows.

TABLE 3.

Neutralization of haemolytic antibody in immune rabbit sera by graded amounts of homologous and heterologous extracts.

(Detailed results of two tests out of a total of fifteen.)

Rabbit serum prepared against		Human	Bovine	Human	Bovine	Human	*Myco.*	Human	*Myco.*
Erythrocytes sensitized with extract prepared from		TB	TB	TB	TB	TB	*phlei*	TB	*phlei*
Serum neutralized with extract prepared from		Human TB		Bovine TB		Human TB		*Myco. phlei*	
		Reciprocal of final serum dilution giving complete haemolysis							
Dilution of extract used for neutralization	∞	6,400	160	6,400	160	6,400	6,400	6,400	6,400
	2^{-10}	3,200	160	3,200	160	3,200	3,200	3,200	3,200
	2^{-8}	1,600	80	1,600	80	1,600	3,200	3,200	1,600
	2^{-6}	400	20	800	20	400	3,200	3,200	400
	2^{-4}	<100	<5	200	5	<100	1,600	3,200	100
	2^{-2}	<100	<5	100	<5	<100	1,600	3,200	<100
	2^{0}	<100	<5	<100	<5	<100	800	1,600	<100
Dilution of neutralizating extract capable of inhibiting 2MHD's of antibody		2^{-8}	2^{-7}†	2^{-8}	2^{-7}†	2^{-8}	2^{-4}	2^{0}	2^{-8}

The overlap between the erythrocyte adsorbable antigenic components of single strains of six species of acid-fast organisms—human, bovine, murine and avian types of tubercle bacilli, Johne's bacillus and *Myco. phlei*—was tested. Two strains were compared in each experiment. The detailed results of two of these fifteen experiments are shown. The summarized results of the cross-neutralization tests are given in Table 4.

† Calculated by interpolation.

Eight doubling dilutions of both absorbed sera were prepared in bulk, the range having been adjusted by preliminary experiment so that complete haemolysis would be obtained in the first five to seven serum dilutions. Five-drop lots of the dilutions of each serum were dispensed in 13 parallel rows of Wassermann tubes. Five fourfold dilutions of the two extracts were prepared in methanol-phosphate-saline extracting fluid (Middlebrook and Dubos, 1948),

ranging from undiluted extract to 1:1,024. One drop of the diluent was added to the tubes of the first rows of the dilutions of both sera. The tubes of the second rows received a drop of the 1:1,024 dilution of one extract, and those of subsequent rows, up to the seventh, a drop each of the decreasing extract dilutions. The tubes of the eighth to thirteenth rows of the dilutions of both sera were treated with single drops of increasing strengths of the other extract in similar fashion. The tubes were shaken and allowed to stand on the bench for 15 to 30 minutes. One drop of a 2·5 p.c. suspension of cells sensitized with one extract was now added to each tube containing dilutions of the homologous serum; the other tubes received one drop of cells coated with the other extract. Controls for the cells were included. The racks were shaken and incubated at 37° C. for 30 minutes. One drop of 1:3 absorbed complement was then mixed with the contents of each tube and the racks placed in the 37° C. water-bath for 45 minutes. The tubes were then read for haemolysis.

The protocols of two of these tests are given in detail in Table 3 and will serve as examples of the types of results obtained. In order to facilitate comparison of the neutralization patterns, the dilutions of neutralizing extracts capable of inhibiting two minimal haemolytic doses of antibody were calculated, where necessary, by interpolation, and set out separately (Table 4). In each square, the value obtained for inhibition of the homologous antibody was placed in brackets following the figure obtained for the inhibition of the heterologous serum, and used as a measure of the neutralizing potency of the extract. A difference in the powers of 2 between the two figures in each square therefore indicated a difference in the neutralization of the homologous and heterologous antibodies by the extract; the antibody against which the value with the higher negative power was obtained had been neutralized by less extract, indicating closer antigenic relationship.

TABLE 4.

Dilutions of extracts capable of inhibiting two minimal haemolytic doses of homologous and heterologous antibodies.

Extract prepared from	Serum prepared against					
	Human TB	Bovine TB	Murine TB	Avian TB	Johne's bacillus	*Myco. phlei*
Human TB	—	2-7 (2-8)	2-7 (2-8)	2-9 (2-8)	2-9 (2-8)	2-4 (2-8)
Bovine TB	2-8 (2-7)	—	2-8 (2-8)	2-8 (2-8)	2-8 (2-6)	2-4 (2-9)
Murine TB	2-4 (2-4)	2-6 (2-8)	—	2-8 (2-8)	2-7 (2-8)	>20 (2-6)
Avian TB	2-5 (2-7)	2-6 (2-6)	2-5 (2-6)	—	2-6 (2-6)	2-2 (2-6)
Johne's bacillus	2-6 (2-8)	2-4 (2-6)	2-4 (2-7)	2-6 (2-8)	—	2-6 (2-8)
Myco. phlei	20 (2-8)	2-4 (2-8)	>20 (2-8)	2-2 (2-8)	2-4 (2-8)	—

The first figure in each square is the result obtained against the heterologous serum. The second (in brackets) is that given against the homologous antibody. For the derivation of the values, see text.

Owing to the nature of the test, a difference of at least two units in the powers of 2 was adopted as the lower limit of significant antigenic difference. It then appeared that (1) there was no significant antigenic difference between the four types of tubercle bacilli tested; (2) extracts of any of the tubercle bacilli were capable of completely inhibiting the haemolytic antibody in the serum prepared against Johne's bacillus, but extracts of the latter organism were deficient when matched against the anti-tubercle sera; (3) there was only slight antigenic overlap between *Myco. phlei* and the other acid-fast organisms tested; possibly the tubercle bacilli were further removed from the saprophyte than was Johne's bacillus.

The six strains therefore fell into three groups: the warm-blooded types of the tubercle bacillus, Johne's bacillus and *Myco. phlei*.

Neutralizing effect of Myco. phlei *extract on haemolytic antibody against cells sensitized with extract of tubercle bacilli of the human type.*

The six rabbit sera and four pools of bovine sera, from the groups of immunized cattle were tested. Each serum was diluted in duplicate rows. To the tubes of one row, one drop of a 1:2 dilution in saline of *Myco. phlei* extract was added; to the tubes of the control row, one drop of a 1:2 saline dilution of the extracting fluid. The tubes were shaken and allowed to stand on the bench for 15 minutes. Cells sensitized with the extract of tubercle bacilli of the human type were then added, the racks incubated at 37° C., complement added, and after further incubation the tests read in the usual way.

TABLE 5.

Neutralization by Myco. phlei *extract of haemolytic antibody against erythrocytes sensitized with extract of tubercle bacilli of the human type in immune sera.*

Source of Serum	Immunizing agent	Haemolytic titre	
		Without addition of *Myco. phlei* extract	With addition of *Myco. phlei* extract
Rabbit	Human TB	5,120	2,560
Rabbit	Bovine TB	2,560	1,280
Rabbit	Murine TB	2,560	1,280
Rabbit	Avian TB	10,240	5,120
Rabbit	Johne's bacillus	10,240	2,560
Rabbit	*Myco. phlei*	2,560	80
Bovine	Human TB	1,280	640
Bovine	Bovine TB	320	160
Bovine	Avian TB	640	320
Bovine	Johne's bacillus	640	160

TABLE 6.

Antibodies against erythrocytes sensitized with extract of tubercle bacilli of the human type in sera of rabbits immunized with Myco. ulcerans, *and in sera of lepers and controls.*

Source of serum	Immunization or clinical condition	Agglutinating titre	Haemolytic titre	Haemolytic titre with addition of *Myco. phlei* extract
Rabbit No. 443	Pre-immunization sample	Not determined	10	Not determined
Rabbit No. 443	Immunized with *Myco. ulcerans*	Not determined	800	20
Rabbit No. 447	Pre-immunization sample	Not determined	<5	Not determined
Rabbit No. 447	Immunized with *Myco. ulcerans*	Not determined	1,600	100
Aborigine	Leper	80	160	80
Aborigine	Leper	80	2,560	20
Aborigine	Leper	640	2,560	320
Aborigine	Leper	1,280	5,120	640
Aborigine	Leper	160	2,560	80
Aborigine	Leper	Irregular	320	40
Aborigine	Non-leprous control	<5	5	Not determined
Aborigine	Non-leprous control	<5	<5	Not determined
Aborigine	Non-leprous control	<5	5	Not determined
Aborigine	Non-leprous control	<5	<5	Not determined
Aborigine	Non-leprous control	<5	20	Not determined

The extract of *Myco. phlei* neutralized one minimal haemolytic dose in each of the sera from animals immunized with tubercle bacilli; two doses in the anti-Johne sera; and sixteen minimal haemolytic doses in the anti-phlei serum (Table 5).

Tests on sera of rabbits immunized with Myco ulcerans *and on sera of lepers.*

These tests were carried out against erythrocytes sensitized with extract of tubercle bacilli of the human type. The degree of reduction of haemolytic titre by extract of *Myco. phlei* was also determined.

The results are shown in Table 6. The sera of the immunized rabbits gave haemolytic titres comparable with titres of sera from animals immunized with the other acid-fast organisms (see Table 1); there was little or no antibody in the pre-immunization bleedings. All of the six sera from lepers contained agglutinating and haemolytic antibodies in high titres; sera from the five non-leprous controls showed little or none.

Addition of the extract of *Myco phlei* to the rabbit sera neutralized sixteen and eight units of haemolytic antibody, respectively; in the case of the sera from the lepers, the inhibition varied between one and sixty-four minimal haemolytic doses.

DISCUSSION.

Strong cross-reactions in haemagglutination and haemolytic tests have shown that antigenic overlap exists between erythrocyte-adsorbable fractions of Mycobacteria as widely separated as virulent tubercle bacilli of the human type and the timothy-grass bacillus. The degree of the overlap between single strains of six species was estimated by means of a neutralization technique which revealed that the four warm-blooded types of tubercle bacilli formed a homogeneous group the members of which shared only a small proportion of the antigenic material with *Myco. phlei;* Johne's bacillus occupied an intermediate position. These six strains thus fell into three groups. Division of the strains into the same groups was indicated by the degree to which the addition of extract of *Myco. phlei* to the immune sera inhibited the haemolytic antibody against cells coated with extract of tubercle bacilli of the human type.

Sera from patients suffering from leprosy, and from rabbits immunized with *Myco. ulcerans* were also found to contain antibody against sheep erythrocytes coated with extract prepared from tubercle bacilli of the human type. The degree of neutralization of the haemolytic antibody by the addition of extract of *Myco. phlei* to the sera of rabbits immunized with *Myco. ulcerans* suggested that the latter organism occupied a place between Johne's bacillus and *Myco. phlei* with respect to structure of erythrocyte-adsorbable antigenic material. When the sera from lepers were tested in the same way, considerable variations were observed in the degree of neutralization. The cause of these variations is not clear.

Antigenic relationships of *Mycobacteria* have been investigated in the past by a number of workers using conventional techniques. In agglutination tests, for instance, cross-reactions were observed throughout the genus; by absorption of agglutinins, tubercle bacilli of the mammalian (human, bovine and murine) types formed a homogeneous group which was distinct from the avian type,

as well as from saprophytes (Wilson, 1925; Griffith, 1925; and Wells, 1946). Avian and mammalian types of the tubercle bacillus could also be distinguished by the precipitin test (Grünberg, 1935). Insofar as a comparison could be made, the haemolytic technique gave results which were in good general agreement with those obtained by the conventional methods, although it failed to differentiate between the avian and mammalian types of tubercle bacilli. Owing to the fact, however, that only a small number of strains was tested, no definite deductions could be made regarding the details of the distribution of the erythrocyte-adsorbable antigenic material throughout the genus.

SUMMARY.

The presence of common antigenic components between the erythrocyte-adsorbable fractions of the four warm-blooded types of the tubercle bacillus, Johne's bacillus and *Myco. phlei* was demonstrated by a haemagglutination and haemolytic technique. One strain of each species was tested.

It was shown by graded adsorption tests that the four types of tubercle bacilli formed a homogeneous group. There was only slight antigenic overlap between the members of this group and *Myco. phlei*. Johne's bacillus occupied an intermediate position.

Antibody against cells sensitized with material extracted from tubercle bacilli of the human type was found in the sera of six aborigine lepers, but not in the sera of non-leprous controls. Antibody against the same antigen was also present in the sera of rabbits immunized with *Myco. ulcerans*.

Acknowledgments. **I wish to thank Dr. E. V. Keogh and Dr. E. A. North for their helpful advice and criticism. Dr. J. J. Graydon carried out the statistical calculation.**

REFERENCES.

Fisher, S. (1950): Austral. J. exp. Biol., 28, p. 613.
Fisher, S. and Keogh, E. V. (1950): Nature, 165, p. 248.
Griffith, A. S. (1924–1925): Tubercle, 6, p. 417.
Grünberg, B. (1935): Z. Tuberk., 73, p. 197.
MacCallum, P., Tolhurst, J. C., Buckle, G. and Sissons, A. H. (1948): J. Path. Bact., 60, p. 93.
Middlebrook, G. and Dubos, R. J. (1948): J. exp. Med., 88, p. 521.
Wells, A. Q. (1946): Med. Research Council Special Report No. 259.
Wilson, G. S. (1925): J. Path. Bact., 28, p. 69.

THE EFFECT OF ATEBRIN ON REDUCTION OF HAEMIGLOBIN (METHAEMOGLOBIN)[1] IN RAT ERYTHROCYTES

by E. C. FOULKES[2]

(From the Institute of Medical Research, the Royal North Shore Hospital, Sydney).

(Accepted for publication 24th July, 1950.)

The ability of blood to reduce haemiglobin to haemoglobin has been known for many years (e.g. Lewin, 1895). Later workers observed that the metabolism particularly of glucose and lactic acid in the erythrocyte was connected with haemiglobin reduction. The kinetics of haemiglobin reduction were investigated in greater detail by Kiese (1943) who found that the enzyme system reducing haemiglobin by means of glucose-6-phosphate consists not only of glucose-6-phosphate dehydrogenase (Zwischenferment) with its specific coenzyme, triphosphopyridine nucleotide (TPN), but of a further enzyme which he named haemiglobin reductase. Haemiglobin reductase oxidizes reduced TPN and reduces haemiglobin to haemoglobin. It is thus fully analogous to cytochrome *c* reductase (Haas, Horecker, Hogness, 1940) and diaphorase (v. Euler and Hellström, 1938; Dewan and Green, 1938) both of which transport electrons from reduced phosphopyridine nucleotides to ferricytochrome *c*. Kiese suspects that like cytochrome *c* reductase and diaphorase, haemiglobin reductase is a flavoprotein.

The red cell enzymes involved in the reduction of haemiglobin have recently been investigated also by Gibson (1948). He obtained evidence that the factor which is absent from the blood of patients suffering from idiopathic haemiglobinemia could be replaced by coenzyme factor I added in the form of a solution containing the Straub flavoprotein (Straub, 1939). Although Gutmann, Jandorf and Bodansky (1947) observed direct reduction of haemiglobin by chemically reduced coenzyme I in the presence of methylene blue, the experiments of Gibson confirm that the direct oxidation of reduced coenzyme I by methylene blue is considerably slower in the absence than in the presence of flavoprotein. Similarly the interaction of reduced TPN and methylene blue is negligible. The accelerating action of dyes such as methylene blue on the reduction of haemiglobin by TPN-dehydrogenases is ascribed by Gibson to the stage between coenzyme factor II and haemiglobin. This is in full agreement with Kiese. Coenzyme factor II corresponds to haemiglobin reductase.

The present investigation was undertaken in the hope of gaining information about the nature of haemiglobin reductase. In particular it was of interest

1 Throughout this paper the nomenclature used is that suggested by R. Lemberg and J. W. Legge in "Hematin Compounds and Bile Pigments," Interscience Publishers Inc., New York and London, 1949.

2 This work was supported by a grant from the National Health and Medical Research Council of Australia.

to determine whether or not it is a flavoprotein. So far the enzyme has not been obtained in a concentrated form.

The inhibition of flavoproteins by atebrin has been studied by various workers (Haas, 1944; Hellerman, Lindsay and Bovarnick, 1946; Madinaveitia, 1946). Although several flavin enzymes are inhibited by atebrin, it is by no means a specific inhibitor. The experiments here reported describe the effect of atebrin on haemiglobin reduction.

Materials and Methods.

With the exception of some preliminary experiments, rat erythrocytes were used throughout as source of the reductase. Blood was collected freshly each day by cardiac puncture from young albino rats of both sexes of the Wistar strain, weighing 120–150 gm. The erythrocytes were washed free from anticoagulant (citrate or oxalate) and haemolysed with toluene. Three volumes of water or 40 p.c. saturated ammonium sulphate were added and the greater part of the haemoglobin crystallized out as carboxyhaemoglobin after saturation with coal gas. The supernatant was used directly, or after dialysis, as source of the reductase. As blood from different animals was used each day, the various preparations of reductase are not comparable. The rate of haemiglobin reduction was approximately proportional to the volume of haemolysate, i.e. the amount of reductase added, but variations between different experiments may have been introduced by other changes in experimental conditions. Only experiments collected in the same Table are thus comparable. Haemiglobin was obtained by autoxidation at pH 5·5 under reduced oxygen pressure, or else by nitrite oxidation of thrice-recrystallized horse oxyhaemoglobin, prepared by the method of Heidelberger (1922). Haemiglobin was estimated photometrically by the method of Evelyn and Malloy (1938) using a Spekker photoelectric colorimeter with red filter H 558 No. 608. The glucose-6-phosphate used was either a pure synthetic sample, or else was in the form of Embden's ester prepared from brewers' bottom yeast by the method of Dubois and Potter (1943). TPN was prepared from human erythrocytes by the method of Warburg, Christian and Griese (1935). It was not purified past stage two. Zwischenferment was prepared from top brewers' yeast by precipitation from Lebedev juice at pH 4·6. Atebrin, 5-aminoacridine and riboflavin were the pure chemicals. Streptomycin was used as its pure sulphate (Pfizer). Carbon monoxide was prepared from sulphuric and formic acids. Nile Blue was the commercial dye (Coleman & Bell Co., Norwood O., U.S.A.).

EXPERIMENTAL.

Table 1 shows that methylene blue does not react directly with reduced TPN.

TABLE 1.

Oxidation of reduced TPN by dyes.

Substrate	Dye	Atmosphere	Decolouration time (minutes)
Glucose-6-phosphate	Methylene blue	CO	∞
—	Methylene blue	CO	∞
Glucose-6-phosphate	Indophenol	air	6
—	Indophenol	air	∞

Each tube contained 1 mg. Zwischenferment, 20 μg. TPN, 10 μg. methylene blue or 2:6-dichlorophenolindophenol; pH 7 (0·1 M phosphate).

Total vol. 0·6 ml. Temperature 37° C. Glucose-6-phosphate added: 1 mg.

That there is little oxidation of reduced TPN by haemiglobin in the absence of haemiglobin reductase is shown in Table 2.

TABLE 2.

Action of haemiglobin reductase.

Additions	mg. haemiglobin reduced per hour at 37° C.
1. Zwischenferment system + reductase	40
2. Zwischenferment system only	0
3 Reductase only	2

Each tube contained 40 mg. haemiglobin, 5 μg. Nile blue; pH 7. Atmosphere CO. Total volume 1·6 ml.

Zwischenferment system: 1 mg. glucose-6-phosphate, 1 mg. Zwischenferment, 20 μg. TPN.

Reductase: 0·75 ml. toluene haemolysate of horse cells after removal of CO-haemoglobin and dialysis.

The reductase-containing haemolysate has appreciable Zwischenferment activity, so that on the addition of glucose-6-phosphate, TPN, dye and haemiglobin, vigorous haemiglobin reduction occurs. Zwischenferment was usually added, however, in order to make the haemiglobin reductase the only limiting factor.

The action of atebrin on the haemiglobin reductase system is illustrated in Table 3; marked acceleration is caused by atebrin in presence or absence of excess TPN. The action of nicotinamide is discussed below. Atebrin was used in roughly the optimum concentration (0·0004 M). Some inhibition was observed at higher concentrations (e.g. 45 p.c. inhibition at 0·002 M atebrin).

TABLE 3.

Atebrin and haemiglobin reduction in the presence of dye.

Additions	mg. haemiglobin reduced in 2 hours at 37° C.
—	15
0·16 M nicotinamide	14
0·0004 M atebrin	41
0·16 M nicotinamide + 0·0004 M atebrin	20
0·5 mg. TPN	21
0·5 mg. TPN + 0·0004 M atebrin	41

Each tube contained 54 mg. haemiglobin, 1 mg. Zwischenferment, 1 mg. glucose-6-phosphate, 20 μg. TPN, 10 μg. Nile blue.

0·7 ml. reductase from rat cells. pH 7·3. Total volume 2 ml. Atmosphere CO.

Atebrin similarly activated haemiglobin reduction if the Nile blue was replaced by methylene blue.

In the absence of dye, the limiting step in the reduction of haemiglobin becomes the electron transfer from reduced haemiglobin reductase to haemiglobin. It is shown in Table 4 that under these conditions atebrin has no appreciable effect.

In the absence of added Zwischenferment, but in the presence of dye, atebrin still exerts its accelerating action (Table 5).

TABLE 4.

Atebrin and haemiglobin reduction in the absence of dye.

Additions	mg. haemiglobin reduced in 2 hours at 37° C. Expt. 1	Expt. 2
—	15	11
0·0004 M atebrin	12	11

Each tube contained 54 mg. haemiglobin, 1 mg. Zwischenferment, 1 mg. glucose-6-phosphate, 20 μg. TPN, expt. 1, 0·3 ml., expt. 2, 0·5 ml. reductase from rat cells. Total vol. 1·1 ml. Atmosphere CO, pH 7·3.

TABLE 5.

Action of atebrin in absence of added Zwischenferment.

Addition	mg. haemiglobin reduced in 2 hours at 37° C.
—	23
0·0004 M atebrin	34

Each tube contained 40 mg. haemiglobin, 20 μg. TPN, 4 μg. Nile blue, 0·8 mg. glucose-6-phosphate, 0·3 ml. reductase from rat cells; total vol. 1·0 ml. Atmosphere CO, pH 7·3.

The effect of various other substances was compared with that of atebrin: (1) streptomycin, a strong base like atebrin, but not sterically related to it; (2) 5-aminoacridine, a strong base sterically related to atebrin; (3) riboflavin, a weak base sterically related to atebrin. The results are shown in Table 6. Obviously only the compounds sterically related to atebrin accelerate haemiglobin reduction.

TABLE 6.

Comparison of atebrin with other compounds.

Exp.	Additions	mg. haemiglobin reduced in 2 hours at 37° C.
1	0·0004 M streptomycin	19
	0·0004 M streptomycin + 0·0004 M atebrin	31
	0·0004 M atebrin	31
2	—	27
	0·0004 M atebrin	46
	0·0007 M riboflavin	48
	0·0004 M 5 —aminoacridine	35
3	—	23
	0·0004 M atebrin	33
	0·0005 M riboflavin	33
	0·0004 M 5-aminoacridine	31

Each tube contained 54 mg. haemiglobin, 1 mg. Zwischenferment, 1 mg. glucose-6-phosphate, 20 μg. TPN. Atmosphere CO, pH 7·3.

Exp. 1. 0·25 ml. reductase from rat cells, 10 μg. Nile blue; total vol. 1 ml.
Exp. 2. 0·5 ml. reductase from rat cells, 5 μg. Nile blue; total vol. 1·4 ml.
Exp. 3. 0·4 ml. reductase from rat cells, 10 μg. Nile blue; total vol. 1·3 ml.

In the absence of added glucose-6-phosphate, atebrin has little effect on haemiglobin reduction (Table 7).

TABLE 7.

Action of atebrin in absence of substrate.

Addition	mg. haemiglobin reduced in 1 hour at 37° C.
—	6
0·0004 M atebrin	9
1·5 mg. glucose-6-phosphate	10
1·5 mg. glucose-6-phosphate + 0·0004 M atebrin	30

Each tube contained 40 mg. haemiglobin, 0·8 mg. Zwischenferment, 16 μg. TPN, 5 μg. Nile blue, 0·3 ml. reductase from rat cells (non-dialysed). Total vol. 1·0 ml. pH 7·3, atmosphere CO.

DISCUSSION.

Neither atebrin nor 5-aminoacridine can form an artificial hydrogen carrier of the type obtained by Albert and Falk (1949) from various acridine and quinoline compounds (J. E. Falk, private communication). A possible explanation of the accelerating action of these acridines is that both combine with an inhibitor of haemiglobin reduction by virtue of their strong basicity. Silverman and Evans (1943) found various basic substances to compete with atebrin and to antagonize its toxic effects on the growth of *E. coli*, whilst Massart (1948) observed streptomycin antagonism to basic bactericidal substances. Accordingly the effect of streptomycin on the haemiglobin reductase system was tested (see Table 6). The fact that the weak base riboflavin stimulates haemiglobin reduction while the strong base streptomycin does not, clearly shows that it is the steric similarity to atebrin and not the strong basicity which is required for the accelerating action. Of course, the dye used throughout these experiments (Nile blue) is itself a basic polycyclic compound, but Kiese (1943) found unrelated compounds such as 2-methyl-1 : 4-naphthoquinone to have the same action as Nile blue, whereas riboflavin could not replace the dye. Atebrin is only effective at a concentration of well above 10^{-4}M whilst Nile blue exerts a marked effect at a concentration of 10^{-5}M. Therefore the accelerating action of the dye is not related to that of atebrin which cannot act as, or give rise to, a hydrogen carrier. If the accelerating action of atebrin is due to combination with an inhibitor, the experiments reveal little about its nature. Atebrin acceleration was found (a) with various samples of well dialyzed haemiglobin prepared from thrice-recrystallized oxyhaemoglobin; (b) in the absence of added Zwischenferment (Table 5); (c) with pure synthetic glucose-6-phosphate as well as with hexosemonophosphate prepared from yeast; (d) in the presence or absence of excess TPN; (e) with Nile blue or methylene blue. The inhibitor would therefore have to be present in the reductase-containing haemolysate. Prolonged dialysis of the haemolysate had no effect on atebrin acceleration.

This also indicates that atebrin does not accelerate haemiglobin reduction by a substrate other than glucose-6-phosphate. In the absence of glucose-6-phosphate atebrin does not accelerate haemiglobin reduction (Table 7). In the absence of added dye, where the reduction of haemiglobin by the reductase becomes the limiting step, atebrin has no accelerating action (see Table 6).

Atebrin acceleration of the haemiglobin reductase system is not similar to its action on yeast carboxylase studied by Silverman (1949). Here atebrin was found to stimulate CO_2 production by inhibiting the dephosphorylation of cocarboxylase by yeast phosphatase. That a similar inhibition of coenzyme destruction does not explain the action of atebrin on haemiglobin reduction can be seen from Table 3. The stimulation of haemiglobin reduction by atebrin was not abolished in the presence of a large excess of TPN, nor did addition of nicotinamide, known to prevent the destruction of pyridine nucleotides by an erythrocyte nucleosidase (Gutmann *et al.*, 1947), cause an acceleration of haemiglobin reduction. The reason for the relative inactivity of atebrin in the presence of nicotinamide is not clear.

In agreement with Haas (1944) it was found that atebrin inhibits the Zwischenferment system. The addition of haemolysate does not interfere with the atebrin inhibition of Zwischenferment activity as measured by the reduction of 2 : 6-dichlorophenolindophenol.

In conclusion, the accelerating action of atebrin on haemiglobin reduction can be attributed either to an effect on the hydrogen transfer from reduced TPN to haemiglobin reductase, or to its effect on the rate of reactions of Nile blue with either reductase or haemiglobin. No information about the nature of haemiglobin reductase can be gained from atebrin inhibition experiments.

SUMMARY.

Atebrin (4×10^{-4}M) does not affect the rate of reduction of haemiglobin by the glucose-6-phosphate dehydrogenase-TPN-haemiglobin reductase system, and even accelerates the reduction in the presence of Nile blue or methylene blue. In the presence of these dyes, compounds sterically related to atebrin (5-amino-acridine, riboflavin) have a similar effect. The mechanism of this acceleration is discussed.

Acknowledgments. My thanks are due to Dr. R. Lemberg for his encouragement in the work. Mr. J. E. Falk kindly supplied the synthetic glucose-6-phosphate and also the riboflavin, 5-amino-acridine, and atebrin. The yeast was obtained from Tooth's breweries.

REFERENCES.

Albert, A. and Falk, J. E. (1949): Biochem. J., 44, p. 129.
Dewan, J. G. and Green, D. E. (1938): *Ibid.*, 32, p. 1,200.
Dubois, K. and Potter, V. R. (1943): J. biol. Chem., 147, p. 41.
Euler, H. v., Hellström, K. (1938): Z. physiol. Chem., 252, p. 31.
Evelyn, K. A. and Malloy, H. T. (1938): J. biol. Chem., 126, p. 655.
Gibson, Q. H. (1948): Biochem. J., 42, p. 13.
Gutmann, H. R., Jandorf, B. J., Bodansky, O. (1947): J. biol. Chem., 169, p. 145.
Haas, E. (1944): *Ibid.*, 155, p. 321.
Haas, E., Horecker, B. L. and Hogness, T. R. (1940): *Ibid.*, 136, p. 747.
Heidelberger, M. (1922): *Ibid.*, 53, p. 31.
Hellerman, L., Lindsay, A., Bovarnick, M. R. (1946): *Ibid.*, 163, p. 553.
Kiese, M. (1943): Biochem. Z., 316, p. 264.
Lewin, J. (1895): Arch. exper. Path. Pharm., 35, p. 401.
Madinaveitia, J. (1946): Biochem. J., 40, p. 373.
Massart, L. (1948): Nature, 162, p. 779.
Silverman, M. (1949): J. biol. Chem., 179, p. 423.
Silverman, M. and Evans, E. A. (1943): *Ibid.*, 150, p. 265.
Straub, F. B. (1939): Biochem. J., 33, p. 787.
Warburg, O., Christian, W., Griese, A. (1935): Biochem. Z., 282, p. 157.

THE DEAMINASE ACTIVITY OF MYOSIN

by G. F. HUMPHREY AND H. L. WEBSTER

(From the Department of Biochemistry, University of Sydney).[1]

(Accepted for publication 15th September, 1950.)

Myosin, once regarded simply as a structural protein, is now known to be enzymically active and able to dephosphorylate adenosinetriphosphate (ATP). In addition to this action, Banga (1947a) has shown that under suitable conditions adenosinediphosphate (ADP) can also be dephosphorylated and further, that ATP and ADP can also undergo deamination.

Adenylic acid deaminase has been studied by Schmidt (1928) who was able to extract it from muscle. More recently Ferdman and Nechiporenko (1946) and Kalckar (1947) have studied this enzyme; these workers have found that myosin deaminates adenylic acid and that a fraction with deaminase activity can be dialysed away at pH 8 from myosin gel. Hermann and Josepovits (1949) showed that the protein component of Schmidt's deaminase is myosin; in addition to other tests they followed the rise in viscosity produced when actin is added to Schmidt's deaminase.

In the present investigation the effects of some activators and inhibitors on the deamination of adenylic acid have been studied.

EXPERIMENTAL.

Material.

Rabbit muscle was used as a source of myosin gel (Bailey, 1942), crystalline myosin (Szent-Györgyi, 1947), protins (Banga, 1947b) and ATP (Needham, 1942).

Adenylic acid (adenosine-5-phosphate) was prepared by the method of Kerr (1941) and contained 19·8 p.c. N and 8·6 p.c. P corresponding to a purity of 98 p.c. Other compounds were obtained from British Drug Houses.

Veronal-acetate buffers were made according to Michaelis (1931) with no added NaCl, and succinate buffer according to Kalckar (1947).

Triple glass-distilled water was used throughout.

Methods.

Activity was calculated as Q_{NH_3}, (μl. NH_3/mg. dry weight/hour) the NH_3 being estimated by direct Nesslerization of a trichloracetic acid filtrate of the experimental mixture. Dry weight of enzyme in salt solutions was calculated after evaporation to constant weight and subsequent ashing; this gave results agreeing with total N (Ma and Zuazaga, 1942) multiplied by the factor 6 (Bailey, 1942) and this latter method was used as a routine.

[1] This investigation was aided by an apparatus grant from the Cancer Research Committee, University of Sydney.

The pH was determined with a glass electrode and the values refer to the mixtures with all components added.

Deamination was also followed by estimating the change in E_{265} either in the reaction mixture or after deproteinizing an aliquot with $HClO_4$; a Beckman, Model DU, spectrophotometer was used with a "grey" filter (Kalckar, 1947).

Results.

Effect of pH.

The change in activity of the enzyme as affected by pH is shown in Fig. 1. The reaction was allowed to proceed for 10 minutes at 37° C. in the presence of 0·006 M veronal-acetate buffer and 2·3 mM adenylic acid. Thrice precipitated myosin gel was used and after stopping the reaction with a final concentration of 8 p.c. trichloracetic acid, ammonia was estimated.

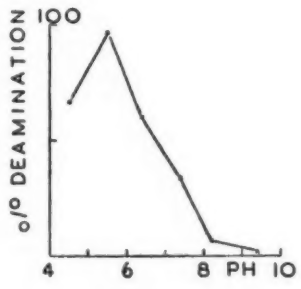

Fig. 1.

Effect of ions.

Small concentrations of KCl, $CaCl_2$ and $MgCl_2$ increase the deaminase activity of myosin preparations. For example (Table 1), myosin gel after the third precipitation was diluted 1:4 with NaCl to give a final NaCl concentration of 0·5 M and incubation carried out at 37° C. in the presence of 0·04 M succinate buffer (pH = 5·5) and 2·3 mM adenylic acid. After 10 minutes 15 volumes of 3 p.c. $HClO_4$ were added and the change in E_{265} determined.

TABLE 1.

Effect of ions on deaminase activity.

The figures given are p.c. of control with no added salts.

100 mM KCl	107
10 mM $CaCl_2$	146
1 mM $CaCl_2$	114
0·1 mM $CaCl_2$	111
10 mM $MgCl_2$	140
1 mM $MgCl_2$	116
0·1 mM $MgCl_2$	112

Activity of enzyme preparations.

The Q_{NH_3} of various samples of myosin gel ranged from 1,000 to 1,500 at 37° C. With recrystallized myosin the activities were much higher, one sample giving a value of 4,000; usually the Q_{HN_3} for recrystallized myosin was about 2,500 at 30° C. Maximum activity was given in the presence of 0·01 M $CaCl_2$.

Effect of inhibitors.

Several inhibitors were used on both gel and recrystallized myosin with similar effects. The most effective inhibitors were those reacting with –SH groups; the activity of these inhibitors could be increased by pre-incubation with the enzyme in the absence of substrate. Pre-incubation itself lowered the enzyme activity (Table 2).

TABLE 2.

Effect of sulphydryl inhibitors on myosin.

190 μg. gel protein were pre-incubated 10 minutes at pH 5·5 in the presence of 0·006 M veronal acetate; adenylic acid was then added to give a concentration of 2·3 mM and after 8 minutes the reaction was stopped with trichloracetic acid. The temperature was 37° C. and the final volume 1·1 ml.; the figures are p.c. of incubated control.

Control (not pre-incubated)	180
0·01 M iodoacetate	29
0·001 M phenylmercuricacetate	50
0·001 M iodosobenzoate	59

To attempt to confirm the importance of –SH groups for deaminase action, glutathione was tried as an activator. Myosin gel was consistently activated, sometimes by as much as 110 p.c. (0·003 M glutathione); recrystallized myosin showed activation only after it had been allowed to stand for some days after preparation (Table 3). Because glutathione interferes with the process of direct Nesslerization, NH_3 was estimated according to Conway (1939).

TABLE 3.

Activation by glutathione.

0·1 ml. enzyme and 0·4 ml. solution containing 0·01 M $CaCl_2$, 0·1 M KCl and 0·1 M succinate (pH = 5·5) were incubated with 0·1 ml. H_2O or 0·05 M glutathione for 20 minutes at 30° C.; 1·0 mg. adenylic acid in 0·4 ml. solution at pH 5·5 was then added and incubation continued for another 10 minutes.

	NH_2.N liberated
Myosin gel	24 μg.
Myosin gel + glutathione	34 μg.
Recrystallized myosin	25 μg.
Recrystallized myosin + glutathione	30 μg.

Myosin also functions as an ATP-ase but with a more alkaline pH optimum; to determine if myosin attaches itself to adenylic acid and ATP at the same part of the protein, the action of adenylic acid as a possible inhibitor of the ATP-ase action of recrystallized myosin was studied. The reaction was carried out at 30° C. in the presence of 0·01 M veronal acetate (pH = 8·0) and 0·005 M $CaCl_2$. Pre-incubation with adenylic acid was not carried out since this would have lowered the concentration of possible inhibitor; the concentration of ATP was 0·7 mM and of adenylic acid, 3·2 mM. It was found that adenylic acid had no effect on the release of inorganic phosphate.

Action of protins.

Several preparations of these acid-stable proteins were made and the effects observed with them were very variable.

No effect could be demonstrated with recrystallized myosin either by incubation for a definite period and estimating the NH_3 set free or by following the course of the reaction with

measurements of the change in E_{265}. Using myosin gel, increased activity was sometimes shown with ADP protein and on a few occasions with ATP-protin.

For example, 0·2 ml. mixtures of 0·96 mg. myosin gel and either 0·22 mg. ATP protin or 0·06 mg. ADP protin were allowed to stand 15 minutes at 20° C. and then brought to 1 ml. and a final concentration of adenylic acid equal to 2·3 mM and veronal acetate (pH = 5·8) equal to 0·02 M. Controls were also carried out simultaneously; the results are shown in Table 4.

TABLE 4.

Effect of protins on deamination.

NH_3 was estimated after 3 minutes at 37° C. and p.c. deamination calculated.

Myosin	56 p.c.
ATP protin	—2 p.c.
ADP protin	4 p.c.
Myosin + ATP protin	66 p.c.
Myosin + ADP protin	91 p.c.

Other substrates.

Adenosine-3-phosphate showed no liberation of P or NH_3 when incubated with myosin in the pH range 5·5 to 10·0. Adenosine underwent slight deamination ($Q_{NH_3} = 20$) in the pH range 7–9.

DISCUSSION.

The pH optimum of 5·5 found for deamination of adenylic acid by myosin is close to the value of 5·9 given for Schmidt's deaminase (1928). This value is far removed from that regarded as optimal for phosphatase activity of myosin. The effects shown by inorganic ions on deamination (Table 1) indicate that both Ca and Mg can function as activators but that higher concentrations are needed than where myosin is acting as an ATP-ase (cf. Ca, Bailey, 1942).

The activity of myosin gel is slightly lower as a deaminase than as a phosphatase; the Q_{NH_3} values found are higher than those of Ferdman and Nechiporenko (1946) whose results correspond to a Q_{NH_3} of about 500. Crystalline myosin is much more active than the gel and Hermann and Josepovits (1949) have reported a Q_{NH_3} as high as 5,800. The higher activity of crystalline preparations is also reflected in the experiments with glutathione (Table 3) which show that essential –SH groups are not so easily lost from crystals as from the gel. Deaminase activity (Table 2) as well as phosphatase activity (Singer and Barron, 1944) is dependent upon intact –SH groups.

Although it is not known what is the point of attachment of ATP to myosin (Straub and Feuer, 1950) the fact that adenylic acid does not inhibit ATP-ase activity suggests that adenylic acid and ATP do not join on to myosin at the same groups.

Banga (1947b) has summarized and extended existing work upon the acid-stable proteins of muscle, i.e. the so-called "protins". In the presence of

myosin and the appropriate protins, ATP can be converted to inosinic acid. It is not yet possible to separate the ATP-P and ATP-N protins from each other, nor the members of the ADP set. The results shown in Table 4 indicate that crystalline myosin retains its protins more strongly than myosin gel and that these protins can function in aiding deamination of adenylic acid as well as ATP.

Therefore, in comparing the deaminating and dephosphorylating activities of myosin towards adenylic acid and ATP respectively it seems that the two substrates join on to the enzyme at different points and are acted upon at different pH optima. Similar effects with inorganic ions and –SH reagents are observed but not to the same degree. Further, both enzymic processes are aided by the presence of certain acid stable proteins.

SUMMARY.

The Q_{NH_3} (μl. NH_3/mg. dry wt./hr.) for the deamination of adenylic acid by myosin gel is of the order of 1,000 to 1,500; with recrystallized myosin the value is about 2,500.

Deamination is most rapid at pH 5·5 and is accelerated in the presence of 0·01 M $CaCl_2$ or $MgCl_2$.

Reagents such as iodoacetate, iodosobenzoate and phenylmercuricacetate inhibit deamination; the activity of preparations which have aged, can be stimulated by glutathione.

Adenylic acid does not affect the ability of myosin to dephosphorylate ATP.

ADP- and ATP-protins stimulate adenylic acid deaminase activity of myosin gel, but not recrystallized myosin; it is concluded that crystalline myosin retains its structural integrity more easily than myosin gel.

REFERENCES.

Bailey, K. (1942): Biochem. J., 36, p. 121.
Banga, I. (1947a): Hung. Acta. Physiol., 1, p. 82.
Banga, I. (1947b): Z. Vit. Horm. Fermentforsch., 1, p. 301.
Conway, E. J. (1939): Micro-Diffusion Analysis and Volumetric Error. Crosby Lockwood and Son Ltd., London.
Ferdman, D. L. and Nechiporenko, Z. Y. (1946): Ukr. Biochem. J., 18, p. 105.
Hermann. W. and Josepovits, G. (1949): Nature, 164, p. 845.
Kalckar, H. M. (1947): J. biol. Chem., 167, p. 429.
Kerr, S. E. (1941): *Ibid.*, 139, p. 131.
Ma, T. S. and Zuazaga, G. (1942): Ind. eng. Chem. anal. Ed., 14, p. 280.
Michaelis, L. (1931): Biochem. Z., 234, p. 139.
Needham, D. M. (1942): Biochem. J., 36, p. 113.
Schmidt, G. (1928): Z. physiol. Chem., 179, p. 243.
Singer, T. P. and Barron, E. S. G. (1944): Proc. Soc. exp. Biol. Med., 56, p. 120.
Straub, F. B. and Feuer, G. (1950): Biochim. Biophys. Acta, 4, p. 455.
Szent-Györgyi, A. von. (1947): "Chemistry of Muscular Contraction," Academic Press Inc., New York.

OVARIAN CYST CONTENTS AS A SOURCE OF VIRUS INHIBITOR: HISTOLOGICAL, CHEMICAL AND RHEOLOGICAL STUDIES

by E. L. FRENCH[1], G. S. GUNTER[2] AND R. MOTTERAM[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne)

(Accepted for publication 25th August, 1950.)

The demonstration by Burnet, McCrea and Anderson (1947) that the substrate for the influenza virus enzyme is present in a number of glandular mucins, stimulated interest in this laboratory in these complex substances. Our interest in the contents of ovarian cysts as a possible source of virus inhibitor substance arose primarily from the fact that Morgan and King (1943) found a proportion of ovarian cysts having contents rich in blood group substance. An examination of the contents of nine human ovarian cysts by Professor Burnet had shown four of them to contain substantial amounts of a substance inhibiting haemagglutination by influenza B virus (LEE) previously heated to 55° C. for 30 minutes. Others, including some of the typical pseudomucinous type, contained only insignificant amounts of virus inhibitor (Burnet, 1948a).

From some of these cysts McCrea (1949) was able to isolate partially purified substance of high inhibitory activity that has been used in several investigations previously reported. It seemed, therefore, to be of interest to try to define the types of cyst with contents of high inhibitory titre with a view both to exploring the possibility of these being a convenient source of raw material for chemical studies on the inhibitor, and to finding any clues as to the origin of the high inhibitor content. In addition it had become clear that most human mucinous secretions contained inhibitor in amounts roughly proportional to their viscosity, the highest titres being found for example in the dense cervical (uterine) mucus. It was felt that exploratory work on the rheological character of mucinous or pseudomucinous ovarian cyst fluids might provide some leads worth following. None of these objectives has been satisfactorily accomplished but it seemed worth recording the considerable amount of information accumulated on various aspects of the pathology of ovarian cysts. The present paper reports a study of 44 human ovarian cysts received from various Melbourne hospitals during a period of 18 months. After some preliminary work it was decided to record for each cyst the histological character of its wall, the volume of its content, the value obtained for total nitrogen, the titre obtained in inhibitor titration against heated LEE virus and the capacity of the fluid to be drawn out into coherent threads—"Spinnbarkeit".

1 Working under a grant from the National Health and Medical Research Council.

2 Work carried out during the tenure of a Gordon Craig Scholarship of the Royal Australasian College of Surgeons.

Tests for the presence of true mucin were also made but were invariably negative.

The results are most conveniently recorded under the two headings (1) the relation of histological structure to the nature of the cyst contents and (2) a comparison of some of the properties of pseudomucin with the true mucins such as those of the uterine cervix.

MATERIALS AND METHODS.

On arrival at the laboratory (usually within 1 to 24 hours after removal) the cysts were drained of their contents and the volumes determined. Frequently the contents of the different loculi were kept separate. The cyst fluids were stored at 0° C. until required.

Histological examination. The tissue was fixed in 10 p.c. formalin, embedded in paraffin and routinely examined by haematoxylin and eosin staining. Specific staining for mucus was carried out by Mayer's mucicarmine and Gomori's silver nitrate-methenamine methods.

Virus inhibitor titrations. Allantoic fluid virus was pooled and heated at 55° C. for 30 minutes. Standard dilutions of this heated LEE influenza B virus were prepared in saline to give five agglutinating doses with 1 p.c. fowl cells. Anderson, Burnet and Stone (1946) have shown that only a minority of fowls provide cells that give a low non-specific inhibitory titre with normal serum and only cells from suitable fowls were used.

The dilutions of cyst fluid were set out in doubling dilutions in saline over the desired range in 0·25 ml. amounts. An equal volume of standard virus dilution was added, and, after shaking, left to stand for 30 minutes at room temperature. A volume of 1 p.c. fowl red cells was then added and the reading made after allowing the cells to settle completely at room temperature. The end point was taken as the "one plus" degree of partial agglutination that has become standard in this laboratory (Burnet, Beveridge, McEwin and Boake, 1945).

Total nitrogen determination. The nitrogen determinations were made by the micro-Kjeldahl method, using the apparatus of Parnas and Wagner. The period of combustion was 8 hours after the addition of the catalyst, selenium dioxide.

Determination of ACRA titres. The ACRA (Acid-alcohol-congo-red) test has been described in detail by Burnet (1948a). Briefly the test consists of dropping suitable dilutions of mucin to which a little congo red has been added into 70 p.c. ethyl alcohol containing 1 p.c. hydrochloric acid. Intact mucin forms a characteristic pattern.

Determination of "Spinnbarkeit". The apparatus used to determine the Spinnbarkeit values of the fluids has been described by Gunter (1949). The distance to which a thread of material could be drawn under standard conditions before it ruptured, was electrically recorded on a revolving drum.

Mucinase from V. cholerae. The preparation of this reagent and its separation from the receptor destroying enzyme has been described by Burnet (1948b).

Receptor destroying enzyme of V. cholerae. Both the crude agar extracts from strain 4Z and eluates from red cells prepared as described by Burnet and Stone (1947) were used.

Calcium borate buffer. This contains $CaCl_2$ 1·0 gm., NaCl 9·0 gm., H_3BO_4 1·203 gm., $Na_2B_4O_7 \cdot 10H_2O$ 0·052 gm. per litre (Burnet and Stone, 1947).

EXPERIMENTAL.

Histological features, virus inhibitor and total nitrogen content of cysts.

By studying the haematoxylin-eosin stained sections and sections specifically stained for mucus, eight common histological types were recognized in the series. Table 1 gives a brief description of these types and the number of examples of each type found.

TABLE 1.

Type of cyst	Number of examples
1. Serous cystadenoma parvilocular and with chiefly smooth inner surface	14
2. Multilocular papillary serous cystadenoma with much epithelial proliferation	2
3. Parovarian cysts with an epithelium resembling the serous cysts	2
4. Pseudomucinous cystadenoma with varying numbers of loculi and degree of papillary proliferation	21
5. Carcinoma	1
6. Dermoid	1
7. Fibroma with mucoid change	1
8. Ovarian goitre	1

TABLE 2.

Cyst number and loculi	Volume in millilitres	Spinnbarkeit in mm.	Total nitrogen in gm./litre	Heated virus LEE inhibitor	Brief histological description
1a	2,500	nil	14·8	140,000	Grossly papillary
b	400	nil	5·6	16,000	
c	90	nil	8·2	18,000	
d	130	nil	10·1	32,000	serous cystadenoma
2	215	nil	6·57	6,000	
3	2,300	nil	1·13	400	Papillary pseudomucinous cystadenoma
4	720	nil	6·54	8,000	Dermoid
5	100	nil	1·04	8,000	Serous cystadenoma
6	2,200	trace	4·13	2,500	Pseudomucinous cystadenoma
7	330	53	14·77	100,000	Grossly papillary serous cystadenoma
8	3,000	trace	4·0	400	Serous cystadenoma
9	2,150	13	2·0	200	Papillary pseudomucinous cystadenoma
10a	3,200	nil	0·59	10	Serous cystadenoma
b	300	nil	9·84	800	Serous cystadenoma
11a	1,200	18	3·60	800	Papillary pseudomucinous cystadenoma
b	250	nil	0·99	20	Papillary pseudomucinous cystadenoma
12a	400	8	2·3	80	Pseudomucinous cystadenoma
b	1,000	nil	2·7	200	Pseudomucinous cystadenoma
c	50	9			Pseudomucinous cystadenoma
d	100	115			Pseudomucinous cystadenoma
13a	1,200	5	2·9	1,600	Pseudomucinous cystadenoma
b	75	120			Pseudomucinous cystadenoma
14a	850	8	4·0	40	Pseudomucinous cystadenoma
b	200	8	3·2	400	Pseudomucinous cystadenoma
c	720	8	3·3	400	Pseudomucinous cystadenoma
15	420	5	6·4	600	Pseudomucinous cystadenoma
16	450	nil	0·7	300	Serous cystadenoma
17	2,200	nil	0·2	60	Parovarian cyst
18a	1,400	4	1·3	500	Pseudomucinous cystadenoma
b	50	72	2·5	800	Pseudomucinous cystadenoma
20	700	nil		160	Serous cystadenoma
21	580	nil	1·2	200	Serous cystadenoma

TABLE 2 (continued).

Cyst number and loculi	Volume in millilitres	Spinnbarkeit in mm.	Total nitrogen in gm./litre	Heated virus LEE inhibitor	Brief histological description
22a	400	8	3·0	160	Pseudomucinous cystadenoma
b	400	8	0·5	20	Pseudomucinous cystadenoma
23	500	nil		320	Serous cystadenoma
26		3·0	10·2	600	Papillary pseudomucinous cystadenoma
27		nil	7·7	1,280	Serous cystadenoma
28a		nil	5·4	640	Serous cystadenoma
b		nil	7·4	480	Serous cystadenoma
29		nil	10·9	320	Serous cystadenoma
30	130	nil	2·1	640	Parovarian cyst
32	1,600	5	8·9	70	Pseudomucinous cystadenoma
33	3,500	nil	0·6	1,280	Pseudomucinous cystadenoma
35	3,500	nil	13·5	25,600	Pseudomucinous cystadenoma
37			2·4	30,000	Pseudomucinous cystadenoma
38a	1,150		7·3	13,000	Pseudomucinous cystadenoma
b				13,000	Pseudomucinous cystadenoma
40			14·1	19,000	Papillary pseudomucinous cystadenoma
41			2·9	<100	Pseudomucinous cystadenoma
42			11·9	900	Spheroidal cell carcinoma
43			7·5	1,400	Serous cystadenoma
44			11·6	2,000	Papillary pseudomucinous cystadenoma
45				1,200	Fibroma with mucoid change
46			12·4	6,000	Serous cystadenoma
47				6,000	Ovarian goitre
48				300	Pseudomucinous cystadenoma
49			8·1	20,000	Papillary pseudomucinous cystadenoma
50			4·03	300	Pseudomucinous cystadenoma

Table 2 sets out the details of the cysts showing the number of loculi; the volume of fluid obtained from each loculus; the nitrogen content of the cyst fluids, their Spinnbarkeit, virus inhibitor and the histological nature of the cyst wall. It can be seen that there was on occasions variation in the virus inhibitor and nitrogen values of individual loculi in the same cyst. Fig. 1 shows the epithelial surface of two adjacent loculi in cyst number 14 and is an example of the variation in the histological appearance seen in different pieces of the same cyst.

When well developed the serous cysts showed the typical mid-interval appearance of the epithelium of the Fallopian tube; that is, high columnar cells of tubal secretory type with elongated nuclei parallel to the long axis of the cell body and a granular protoplasm which did not stain with mucicarmine or silver-methenamine. These cells alternated with patches of lower ciliated cells with a clear protoplasm and large nuclei at right angles to the long axis of the cell. These also failed to stain with mucus stains (see Fig. 1).

The majority of the pseudomucinous cystadenomata showed extensive papillary developments with tall columnar epithelium having a basal nucleus and translucent cytoplasm containing secretion granules staining with mucicarmine and methenamine-silver. These high columnar cells sometimes alternated with patches of cuboidal and even flattened cells, adjacent loculi often showing this difference of pattern (see Fig. 1), but in all cases the epithelial cells contained the same secretion granules. Occasionally the wall was largely denuded of epithelium

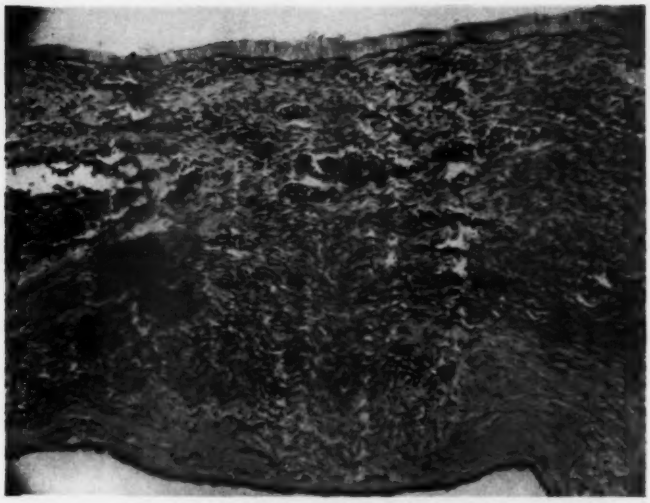

Fig. 1. (Cyst 14). Section showing the contrasting epithelia of adjacent loculi in a pseudomucinous cystadenoma. The upper loculus is lined by typical high columnar "picket fence" epithelium, while the lower loculus shows a flattened epithelium. (H and E × 110.)

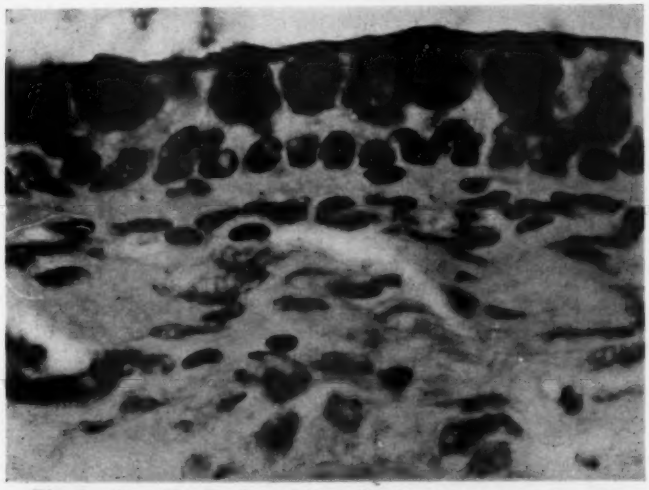

Fig. 2. (Cyst 11). Portion of the epithelium from a pseudomucinous cystadenoma, stained with mucicarmine and haematoxylin. Note the large cells distended with mucus and having a small compressed basal nucleus. These cells have a "goblet" shape but actually are in the process of being shed from the epithelial surface giving a holocrine method of mucus secretion. (850 ×)

and frequently secretion laden cells in the process of being shed were prominent. These cells have been erroneously referred to as goblet cells and their significance will be discussed later.

None of the cyst fluids provided a positive result when tested by the ACRA method even in the undiluted state.

The relationship between pseudomucin and true mucin.

A true mucin may be defined as a macromolecular compound containing polysaccharide and aminoacids which is precipitated from solution by weak acid and other reagents, in the form of a fibrous contractile clot. In addition to acetic acid precipitation, use has been made in this laboratory of the ACRA test and of precipitation by protamine or cetyl trimethyl ammonium bromide solutions (Burnet, 1949a). None of these tests was positive with any of the cysts studied in this series although Burnet (1949b) has described one cyst probably of type 2 in the present classification which gave typical tests for true mucin.

As will be discussed later the histology of the wall of a typical pseudomucinous cyst closely resembles the epithelium of the cervical canal which secretes a true mucin. It was therefore of interest to consider the two types of secretion in relation to each other and the most profitable line of attack seemed to be in regard to the action of enzymes known to influence mucoid substances. In view of the unavailability of precipitation tests the effect of the enzymes on the Spinnbarkeit of those cyst fluids showing a high enough initial value, was tested.

Burnet and Stone (1947) described the production of several enzymes by *V. cholerae* when grown on suitable media. One, the receptor destroying enzyme (RDE) has been shown to split off from ovomucin (Gottschalk and Lind, 1949a), a nitrogen containing glycoside of a composition very similar to that liberated from the same substrate by the influenza virus enzyme (Gottschalk and Lind, 1949b, Gottschalk and Partridge, 1950). Another was found by Burnet (1948b) to have a depolymerizing action on glandular mucin without having any effect on hyaluronic acid.

Many of the cysts contained fluid lacking in Spinnbarkeit (Table 2) but two, viz. Nos. 12 and 13 were found to have fluids with suitable properties. Using these mucins as substrate a series of tests were run with various enzyme preparations.

The activity of an agar filtrate of *V. cholerae* containing no RDE activity was titrated by mixing equal volumes (0·25 ml.) of cyst fluid number 12 with a series of dilution in calcium borate buffer of the enzyme. The mixtures were incubated at 37° C. for 1 hour and after allowing to cool to room temperature, the Spinnbarkeit was determined. A control was included having one volume of calcium borate buffer in place of the enzyme. The end-point was taken as that dilution of the enzyme which reduced the Spinnbarkeit of the fluid to half its original value (Gunter, 1949).

TABLE 3.

Dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	C
Fibre length mm.	3	5	8	10	13	16	19	21	21

Table 3 shows the values obtained with an agar filtrate of *V. cholerae* titrated with cyst fluid number 12d. The fibre half length is reached with a dilution of 1 in 80 of enzyme.

The *V. cholerae* mucinase was prepared free from RDE and a sample of RDE was obtained free from mucinase by the techniques of Burnet (1948b) and Stone (1947).

A dilution of the mucinase before and after heating in a boiling water bath for 15 minutes, and a dilution of RDE was incubated with fluid from cyst No. 12 for 1 hour at 37° C. Table 4 shows the Spinnbarkeit values obtained in each case.

TABLE 4.

Cyst fluid (No. 12) with:		Fibre length (mm.)
Mucinase	1:10	0
Heated mucinase	1:10	26
RDE	1:10	23
Saline (control)		25

The receptor destroying enzyme and the heated mucinase had no effect on the Spinnbarkeit of the mucin but the mucinase preparations reduced the value to zero. This experiment was repeated using fluid from cyst No. 13 as substrate with similar results.

The action of metaperiodate on the heated LEE virus inhibitor (Burnet, 1949b), on the virus receptors of red cells (Fazekas de St.Groth, 1949) and epithelial cells of the lungs (Fazekas de St.Groth and Graham, 1949) has been extensively studied. The reaction of metaperiodate with cyst fluid was tested by allowing freshly prepared concentrations of KIO_4 between M/200 and M/51,200 to react with cyst fluid (No. 13) for 1 hour at 37° C. The mixtures were examined for Spinnbarkeit. Over the whole of this range metaperiodate failed to cause any significant reduction in the fibre length. In one experiment M/300 KIO_4 was allowed to react with cyst fluid overnight without any detectable change in the Spinnbarkeit of the fluid. It is probable, however, that the concentration of metaperiodate was too low in this experiment, and that the periodate was inactivated by the extraneous material present in the undiluted cyst fluid. This view finds support in the results of an experiment in which periodate, acting upon a suitable dilution of true mucin from the human uterine cervix, brought about depolymerization, i.e. destruction of ACRA titre of the mucus as well as destruction of the virus inhibitor content.

Robertson, Ropes and Bauer (1941) described the breakdown of a number of mucins by treatment with ascorbic acid and hydrogen peroxide. This breakdown occurred without liberation of detectable reducing groups and was detected by a fall in the viscosity of the mucins.

Skanse and Sundblad (1943) confirmed and extended the work of Robertson and his coworkers by showing that the viscosity of hyaluronic acid solutions was markedly reduced by ascorbic acid in the presence of atmospheric oxygen. Copper ions increased this effect which was completely inhibited by diethyldithiocarbamate or cyanide. Hydrogen peroxide, while having a slow viscosity-reducing effect itself, was found to greatly increase the effect of ascorbic acid. The decrease in the viscosity was accompanied by a corresponding decrease in the double refraction of flow shown by native hyaluronic acid solution.

The action of ascorbic acid and hydrogen peroxide on cyst fluid was tested. A quantity (0·5 ml.) of cyst fluid (No. 13) was mixed with (a) ascorbic acid alone, (b) hydrogen peroxide alone and (c) ascorbic acid and hydrogen peroxide, and incubated at 37° C. for 1 hour. After cooling to room temperature the Spinnbarkeit titres were determined.

TABLE 5.

Cyst fluid 13	Incubated with:	Fibre length (mm.)
0·5 ml.	0·20 ml. M/100 ascorbic acid and 0·05 ml. water	40
0·5 ml.	0·05 ml. H_2O_2 10 vols. 0·20 ml. water	38
0·5 ml.	0·20 ml. M/100 ascorbic acid, 0·05 ml. H_2O_2 10 vols.	3
0·5 ml.	0·25 ml. water	59

It can be seen from Table 5 that ascorbic acid and hydrogen peroxide together cause almost complete disappearance of Spinnbarkeit while each alone will cause only a small fall in the value.

A number of enzyme inhibitors were tested for their action by incubating 1 vol. of the inhibitor with three volumes of the enzyme preparation for 1 hour at 37° C. One volume of this mixture was incubated with cyst fluid and then tested for Spinnbarkeit.

TABLE 6.

Reaction mixtures	Fibre length (mm.)
Cyst fluid + saline alone	50
Cyst fluid + enzyme treated with saline	8
Cyst fluid + enzyme treated with M/5 calgon	72
Cyst fluid + enzyme treated with 5 p.c. sodium citrate	7
Cyst fluid + enzyme treated with 1 p.c. fluoride	8
Cyst fluid + enzyme treated with 1 p.c. iodoacetic acid	15
Cyst fluid + enzyme treated with 4 p.c. formalin	40
Cyst fluid + enzyme treated by boiling for 15 minutes	44

Table 6 shows that only formalin, calgon, and boiling water had any significant effect upon the enzyme. Neutralized calgon (pH 7·0) was found to increase the fibre length when added to other cyst fluids. It did not, however, produce Spinnbarkeit in a fluid having none to begin with. No further attempt was made to study this phenomenon.

Both RDE and the mucinase are antigenic and by immunizing rabbits with suitable material specific antisera can be obtained. In addition to these two antisera which have been described previously (Burnet and Stone, 1947; Burnet, 1949c), rabbits were immunized with a washed formalized suspension and a washed suspension of *V. cholerae* strain 4Z grown on 0·1 p.c. phenol agar. The former suspension yielded a high titre "H" agglutinating serum and the latter a high titre specific "O" agglutinating serum.

A dilution of 1/10 of each serum was incubated with 4Z filtrate at 37° C. for 1 hour and then this mixture was incubated for another hour at 37° C. with cyst mucin.

TABLE 7.

Reaction mixtures	Fibre length (mm.)
Cyst fluid + saline alone	50
Cyst fluid + enzyme treated with saline	7
Cyst fluid + enzyme treated with anti-mucinase	45
Cyst fluid + enzyme treated with anti-RDE	8
Cyst fluid + enzyme treated with 4Z "H" antiserum	13
Cyst fluid + enzyme treated with 4Z "O" antiserum	8
Cyst fluid + enzyme treated with normal rabbit serum	12

Table 7 shows that anti-mucinase is the only serum significantly inhibiting the action of 4Z filtrate upon the Spinnbarkeit of cyst fluid 13 B. Anti RDE and "H" and "O" agglutinating antisera are without any action on the mucinase.

The relevant characteristics of cervical mucin may be referred to here, and a brief account given of some additional experiments on this substance. The specimens were obtained in the form of very small samples removed from the cervices of patients during examination in the gynaecological department of the hospital.

After weighing, the specimens were dissolved in sterile distilled water to make a 10 p.c. solution in terms of the original. An occasional specimen of the thicker inspissated plug of cervical mucus present during pregnancy was obtained at post mortem. This latter mucus went into solution more slowly but just as completely as that from the non-gravid uterus.

Burnet (1948b) showed that such solutions of cervical mucus gave positive ACRA tests to high dilution and that this character was destroyed by mucinase but not by RDE freed of active mucinase. This was confirmed during the present work. All specimens were found to be rich in virus inhibitor against heated LEE, the titres reached appearing to be roughly related to the density and viscosity of the sample used. Both ACRA titre and the virus inhibitor titre fluctuated according to the phase of the menstrual period at which the sample was taken. This fluctuation appeared to be related to the dilution and change in the rheological character of the mucus occurring normally at the mid-period (Scott-Blair, 1941; Clift, 1945-6).

DISCUSSION.

An examination of Table 2 throws very little light on the question of the origin of the virus inhibitor substances. High values are obtained from two groups of cysts. The first consists of two multilocular and papillary serous cystadenomata (Nos. 1 and 7) and both these cysts show inhibitor titres up to 100,000 and very high nitrogen contents. To some extent at least the inhibitor content and the total N values of serous cyst fluids appeared to depend upon the area of secreting epithelium present. Thus the extensively papilliferous serous cysts (Nos. 1 and 7) showed the highest values for nitrogen and virus inhibitor and those with denuded epithelium the lowest. In cyst 7 there was no ciliated epithelium and there were also some cells staining faintly for mucus amongst the predominantly serous type.

The second group with inhibitor titres over 10,000 contains five pseudomucinous cysts (Nos. 35, 37, 38, 40 and 49). These also show high N values with the conspicuous exception of No. 37. There were many other macroscopically and histologically similar cysts whose contents contained only trivial amounts of inhibitor. A considerable majority (9 out of 14) of the pseudomucinous fluids tested gave Spinnbarkeit values of 5 mm. or more. The two (Nos. 12 and 13) with very high values had only very small amounts of virus inhibitor.

The cells of the epithelium of the pseudomucinous cysts always contained cytoplasmic granules staining with mucicarmine and methenamine-silver and the majority showed considerable papillary development. Sometimes this seemed to be associated with high nitrogen and inhibitor levels, e.g. Nos. 40 and 49, but with at least equal frequency papillary growth was not associated (Nos. 3, 9, 11, 26 and 44 all gave low inhibitor titres and 3, 9, and 11 low values for N.). There seems to be no consistent relationship between type of cyst, and the properties of their contents investigated in this study. Indeed, great variation is found in different loculi of the same cyst (see Fig. 1).

The ovarian cystadenomata are neoplasms and it is possible that the variation in secretory function observed more frequently in neoplastic than in normal epithelium could be responsible for this apparent lack of correlation.

The so-called goblet cells often seen in pseudomucinous cysts have caused some writers to deduce that the epithelium is intestinal in type (Ewing, 1940). In sections these cells are seen to be distended with secretion and are actually in process of being shed into the lumen of the cyst (see Fig. 2).

Thus they do not retain their position in the epithelial surface, nor do they discharge their contents through an opening in the free surface as do true goblet cells but instead are an example of holocrine secretion. Moreover the epithelium of pseudomucinous cysts does not possess a striated border as does true intestinal epithelium. Actually its morphology is closely similar to the epithelium of the cervical canal. However, the secretion of the cervix is a true mucin (it is precipitated as a fibre by acetic acid and gives the ACRA test) whilst pseudomucinous cyst fluid is not a true mucin and fails to give the ACRA test. Burnet (1948a) has described a cyst contents in which he found one loculus containing a thick tenacious mucin that dissolved to give an ACRA positive solution with a high virus inhibitor titre. The other loculi in this cyst were filled with pseudomucin.

The experiments dealing with the destruction of Spinnbarkeit by *V. cholerae* mucinase and by ascorbic acid indicate that in most fluids from pseudomucinous cysts there are more or less highly polymerized molecules which give to the fluids their spinnbar quality. The action of the *V. cholerae* mucinase on the Spinnbarkeit of these cyst fluids is exactly paralleled by the action of this enzyme on guinea-pig intestinal mucus and human cervical mucus (Burnet, 1948b). It is completely neutralized by an anti-mucinase serum but not by an anti-RDE serum, and is not affected by normal serum or agglutinating sera prepared against the "H" and "O" antigens of *V. cholerae* strain 4Z.

The implications are clearly that the mucinase destroys or weakens the groupings responsible for maintaining the long chain macromolecular structures both in true glandular mucins and in ovarian cyst pseudomucin of high Spinnbarkeit. It is equally clear that if the inhibitor quality is carried as seems likely, by the mucin macromolecules in glandular secretions, this quality is quite unrelated to the degree of polymerization.

TABLE 8.

Reagent	Glandular mucin		Hyaluronic acid mucin		Ovarian cyst pseudomucin	
	P.M.	Inhibitor	P.M.	Inhibitor	P.M.	Inhibitor
Nil	+	+	+	—	+	—
V. cholerae RDE	+	—	+	—	+	—
V. cholerae mucinase	—	+	+	—	—	—
H_2O_2 — ascorbic acid	—	—	—	—	—	—

P.M. + = Polymerized molecule, as determined either by ACRA test or Spinnbarkeit, present after treatment with reagent shown in the first column.

Inhibitor — = the inhibitor for treated influenza virus (LEE) is absent after treatment with the reagent shown in the first column.

It is convenient to present a simplified table of the reactions of the three principal types of mucin found in human tissues or secretions; glandular mucin as typified by cervical mucin, hyaluronic acid complexes as typified by synovial fluid, and ovarian cyst pseudomucin. The reagents shown are the two *V. cholerae* enzymes, and hydrogen peroxide-ascorbic acid.

If any conclusion is possible from our data it would probably be that there is some justification for considering pseudomucin as being basically similar to true glandular mucins and with no relation to the hyaluronic acid mucins. It is probably modified in regard both to its degree and character of polymerization, and to the proportion of units which carry the labile amino-acid carbohydrate groupings which, according to the work of Gottschalk and Partridge (1950), may be responsible for virus inhibition and render the mucin a substrate for RDE. What is quite uncertain is whether the pseudomucin is secreted essentially in the same form as it is found when the cyst is removed sometime later, perhaps after the lapse of many years. The possibility that slow enzymic or spontaneous change takes place must be considered. There is a little evidence in Table 2 that in multilocular cysts the smaller, presumably the younger, loculi tend to contain material of higher Spinnbarkeit (cysts 12, 13, 18). There is no such evidence in regard to virus inhibitor and it seems unlikely that secondary processes are responsible for the low titre of most pseudomucinous fluids.

The nature of the virus inhibitor is still uncertain and it is even possible that the invariable association of inhibitor activity with mucoid and mucinous materials may mean merely that the inhibitor is a substance of very high activity present in minute amount in most mucinous secretions, and not, as we have been inclined to think, a property of certain groupings carried by a very wide range of polysaccharide-protein complexes. On either hypothesis there seems to be no satisfactory way to account for the rough correlation between total N content and inhibitor titre in the fluids.

SUMMARY.

Forty-four human ovarian cysts have been examined for their histopathological features, and the contents examined for their rheological properties, for their virus inhibitor content and their total nitrogen levels. There were eighteen serous cystadenomata, twenty-one pseudomucinous cystadenomata and one each of carcinoma, dermoid, fibroma with mucoid change and ovarian goitre.

None of the cyst fluids examined contained a true mucin precipitable by acetic acid with the formation of a firm contractile clot. Several of the pseudomucinous cysts, however, yielded fluids having varying degrees of Spinnbarkeit.

Using two of these fluids as substrate the action of various enzymes, of metaperiodate, and of hydrogen peroxide-ascorbic acid mixtures on the Spinnbarkeit of pseudomucin has been studied. The relationship of pseudomucin to

true glandular mucin is discussed in the light of the results obtained in these experiments.

There appeared to be no relationship between the histological appearance of the cyst walls and the amount of virus inhibitor present in the fluids. There did appear to be a rough correlation between the total nitrogen content and the virus inhibitor level of the various cyst fluids. The possible association of inhibitor molecules with mucoid molecules is discussed.

Acknowledgments. This work was suggested to us by Prof. F. M. Burnet following his observations mentioned in Burnet (1948a). We wish to express our thanks to Prof. Burnet for his encouragement during the work and for much advice during the preparation of the paper. Dr. J. F. McCrea and Miss Sara Weiden of this Institute kindly conducted some of the nitrogen estimations mentioned in the text.

We are indebted to Dr. Bruce Anderson of the Gynaecological Department, Royal Melbourne Hospital for obtaining the specimens of uterine cervical mucus and to all those surgeons and pathologists who have supplied us with the ovarian cysts used in this study.

REFERENCES.

Anderson, S. G., Burnet, F. M. and Stone, J. D. (1946): Austral. J. exp. Biol., 24, p. 769.
Burnet, F. M. (1948a): *Ibid.*, 26, p. 371.
Burnet, F. M. (1948b): *Ibid.*, 26, p. 71.
Burnet, F. M. (1948c): Lancet, i, p. 7.
Burnet, F. M. (1949a): Austral. J. exp. Biol., 27, p. 245.
Burnet, F. M. (1949b): *Ibid.*, 27, p. 361.
Burnet, F. M. (1949c): *Ibid.*, 27, p. 217.
Burnet, F. M., Beveridge, W. I. B., McEwin, J. and Boake, W. (1945): Austral. J. exp. Biol., 23, p. 177.
Burnet, F. M., McCrea, J. F. and Anderson, S. G. (1947): Nature, 160, p. 404.
Burnet, F. M. and Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 227.
Clift, A. F. (1945–6): Proc. Roy. Soc. Med., 39, p. 1.
Ewing, J. (1940): Neoplastic Diseases, 4th ed., p. 667. Saunders, Philadelphia.
Fazekas de St.Groth, S. (1949): Austral. J. exp. Biol., 27, p. 65.
Fazekas de St.Groth, S. and Graham, D. M. (1949): *Ibid.*, 27, p. 83.
Gottschalk, A. and Lind, P. E. (1949a): Brit. J. exp. Path., 30, p. 85.
Gottschalk, A. and Lind, P. E. (1949b): Nature, 164, p. 232.
Gottschalk, A. and Partridge, S. M. (1950): Nature, 165, p. 684.
Gunter, G. S. (1949): Austral. J. exp. Biol., 27, p. 265.
McCrea, J. F. (1949): Unpublished.
Morgan, W. T. J. and King, H. K. (1943): Biochem. J., 37, p. 640.
Robertson, W. V. B., Ropes, M. W. and Bauer, W. (1941): Biochem. J., 35, p. 903.
Scott-Blair, G. W. (1941): Biochem. J., 35, p. 1,039.
Skanse, B. and Sunblad, L. (1943): Acta Physiol. Scand., 6, p. 37.
Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 137.

THE ENZYMATIC ARCHITECTURE OF THE UNFERTILIZED OYSTER EGG

by K. W. CLELAND

(From the Department of Histology and Embryology, University of Sydney).

(Accepted for publication 26th September, 1950.)

In a previous paper (Cleland, 1950b) some aspects of the intermediary metabolism of the unfertilized oyster egg were detailed. A study of the enzymatic architecture of the cell seemed desirable since this knowledge is necessary for a clear formulation of the problems of cell division and differentiation in relation to metabolism (Cleland, 1950a) and would relate the metabolism of the egg to a previous study of cell architecture (Cleland, 1947). Such a study was made in the 1949 and 1950 seasons and forms the basis of this paper.

In recent years cell respiration has been shown to be intimately connected with cell granules. While mitochondria were very early linked with cell metabolism (cf. Cowdry, 1918) a specific connection with respiration was first suggested by Kingsbury (1912) and Mayer *et al.* (1914). Although Warburg (1914) demonstrated that much of the oxidative activity of sea urchin egg *brei* was associated with the granular components, the relevance of the finding to the mitochondrial problem was not appreciated and this type of study lapsed. The mitochondria respiration relation was supported by Joyet Lavergne (1927–38) but none of this work can be considered conclusive.

Studies of centrifugally-broken Arbacia eggs provided a number of suggestive facts. The experiments of Shapiro (1935) indicated that the heavy halves, which contain the pigment granules and about three-quarters of the yolk granules, have a higher endogenous respiration than the light halves, and Ballentine (1940) from his study of the distribution of reducing systems considered that they were associated with the granules. These studies were not extended to quarter eggs in which granule fractionation is more satisfactory.

Hutchens *et al.* (1942) made a study of the distribution of cytochrome oxidase in various fractions separated from sea urchin egg homogenates. They were led to conclude that a considerable fraction was present in the ground cytoplasm where it was also most active in terms of total N. This finding is difficult to reconcile with that of Navez and Harvey (1935) and with more recent reports.

Mitochondria were first separated from cells, and analysed, by Bensley and Hoerr (1934) but seven years elapsed before their respiratory characteristics were examined by Lazarow (1943), Lazarow and Barron (1941). Chantrenne (1944) showed that cytochrome oxidase and succinoxidase are present in the granules separated from yeast and other cells. Schneider (1946a) and Hogeboom *et al.* (1946) demonstrated the presence of succinoxidase and cytochrome oxidase on the mitochondrial fraction. The presence of the tricarboxylic acid cycle and fatty acid oxidase in mitochondria was demonstrated by Kennedy and Lehninger (1949).

Most of the recent work has been done on mitochondria from mammalian liver and little attention has been paid to the general validity of the distribution. However, Recknagel (1950) has shown that cytochrome oxidase is associated with the mitochondrial fraction of the frog's egg, the large yolk granules having little activity, and Crane and Keltch (1949), Keltch *et al.* (1949) have found a number of tricarboxylic acid cycle enzymes associated with a cell-free

enzyme preparation from sea urchin eggs which they state is particulate. This finding is in accord with earlier investigations.

The work of Du Buy *et al.* (1949) has suggested that mitochondria may preserve their enzyme complement on undergoing a cytological differentiation to melanin granules.

In a previous study of oogenesis (Cleland, 1947) two clear-cut granular categories were described:

(a) The P granules (protein yolk) which are spherical, peripherally located in the mature egg, and were thought to arise *de novo* from the cytoplasm of the early oocyte. These granules, both intracellularly and extracellularly, stain intensely with Nile Blue and are fuchsinophilic. In the test-tube they also stain with Janus Green B. In centrifuged eggs they form a thick centrifugal layer with a sharp upper boundary.

(b) The L granules (lipide yolk) which are spherical and located in the central region of the egg. They are composed of phospholipide and neutral fat and in the centrifuged egg form a thick centripetal layer with a sharp lower boundary. In heavily centrifuged eggs the area containing these granules is the first to separate from the cell.

By the use of higher centrifugal forces than were then available, two more granular categories have been recognized:

(c) M granules (mitochondria) which form a thin loose layer, with an irregular upper boundary, above the P granules. They are very small and only just visible microscopically. In suspensions they stain macroscopically with Janus Green B and Nile Blue and appear to be more or less spherical. They are not recognizable in uncentrifuged mature eggs, either living or sectioned, because of their small size and the abundance of other granules present. Even with phase contrast they are not demonstrable in early oocytes, probably because of the high refractive index of the ground cytoplasm, nor has a clear demonstration been possible in sections stained with acid fuchsin. Because granules of similar size may be seen with the phase microscope in the living cells of other oyster tissues and because of their enzymatic and chemical composition they may be homologized with mammalian mitochondria. No successful staining of these granules in fixed smears has been possible; they appear to disintegrate when fixed in osmic acid vapour.

(d) S granules (submicroscopic) are separable from homogenates by high centrifugal forces. Because of their composition and lack of associated enzymes they may be homologized with mammalian microsomes. Like the P and M granules, suspensions stain macroscopically with Janus Green and Nile Blue.

Materials and Methods.

Egg suspensions of *Ostrea commercialis* were prepared as described previously (Cleland, 1950a). They were homogenized either in a ground glass apparatus or in the microcup of a Waring Blendor run at one-third maximum voltage. In both cases the apparatus was surrounded by an ice jacket. The eggs and germinal vesicles were completely cytolysed by the treatment.

The homogenizing medium in the analytical work contained 0·2 M KCl and 0·02 M Na citrate; the pH was 7·5. In the enzymatic studies the medium contained 0·2 M KCl, 0·01 M $MgCl_2$ and 0·01 M phosphate buffer pH 7·5. Practically no aggregation of granules occurred in these media, the suspensions remaining stable for at least 24 hours in the refrigerator. Hypertonic sucrose solutions (Hogeboom *et al.*, 1948) were investigated but appeared to offer no advantage, and, since they require longer centrifugation times, their use leads to greater heating during centrifugation.

The crude homogenates were fractionated in a Servall SSIA centrifuge. No means of refrigerating the centrifuge were available but the heating of chilled homogenates insulated with the thick 15 ml. bakelite adaptors was slight in the times required.

After study with the phase microscope of the fractions obtained from varying combinations of time and centrifugal force, the following schedule was adopted:

(a) *P granules*: centrifuged at 5,000 × *g* for 5 minutes. Precipitate washed twice in the analytical studies and once in the enzyme studies.

(b) *M granules*: combined supernatants from (a) centrifuged at 10,000 × *g* for 10 minutes. Precipitate washed once.

(c) *S granules*: combined supernatants from (b) centrifuged for 30 minutes at 20,000 × *g*. Precipitate washed once.

The L granules form a white cap on the supernatants. Since some adhered to the sides of the tubes there was a small contamination of all fractions with L granules. As the centrifugal and analytical studies indicated that these granules were pure lipide this contamination is unimportant except that it vitiates phospholipide analyses. Apart from this, and a contamination of the P granules with nucleoli, the fractions appeared to be pure under the phase microscope.

The supernatant from (c) was treated with trichloracetic acid to a final concentration of 10 p.c. and precipitate and supernatant preserved for analysis.

Total N was determined by direct Nesslerisation of $H_2SO_7/HClO_7$ digests. Ribonucleic acid was determined on whole granule suspensions with the McRary and Slattery (1945) reagent, the coloured derivative being extracted with amyl alcohol before measurement in the Spekker photoelectric absorptiometer.

Manometric and other methods were the same as described previously; 5 ml. microflasks were used in all the quoted experiments. Spectroscopic investigations were made with a Zeiss microspectroscope.

Non-respiratory enzymes were studied by methods previously described (Cleland, 1950b).

RESULTS.

Composition of granules.

The analyses shown in Table 1 indicate that most of the particulate nitrogen is present in the P granule fraction. The amount recovered in the M granule fraction is higher than would be expected from microscopic observation of centrifuged whole eggs. Since there is a colour difference between P and M granules it is unlikely that this disparity is due to contamination of the M granule fraction by P granules; it is probably due to the invisibility of a proportion of the M granules in the centrifuged living egg. It will be seen that the nucleic acid content increases with decreasing size of granule and is highest in the soluble ground cytoplasm, a finding expected from previous histochemical studies (Cleland, 1947). In the sea urchin egg also, the maximum concentration of nucleic acid is in the ground cytoplasm (Harvey and Lavin, 1944).

TABLE 1.

Distribution and composition of fractions.

	Whole homogenate			P granules			M granules			S granules			Ground cytoplasm		
Expt.	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	100	100	15	39	14	7·6	4·6	3·6	12·2	3·9	3·8	16	33	52	24·5
2	100	100	14·5	30	14	5·8	4·5	2·5	8·8	3·6	3·0	14·4	41	60	23
3	100	100	15·3	33	12	7·4	2·9	2·1	11·6	2·9	2·8	16	38	50	20
4	100	100	13·6	21	8·5	6·8	5·1	2·5	8·0	4·0	2·6	11·3	50	62	19
Mean	100	100	14·6	30	12	6·8	4·3	2·7	10	3·6	3·3	16	41	56	21
Colour	—			light reddish grey			white			white			—		

The figure in column 1 is the percentage of the total nitrogen recovered in each fraction, column 2 the p.c. of the total pentose and column 3 the p.c. of nucleic acid in the protein of the fraction (calculated from pentose and protein N figures). The ground cytoplasm is the fraction precipitable by trichloracetic acid from the supernatant of the S granule fraction.

Succinoxidase activity.

In Table 2 the succinoxidase activity of granule preparations is given. It will be seen that the enzyme is present in both the P and M fractions, the latter being the more active, and that very little activity is demonstrable in the soluble supernatant. It is presumed that both P and M granules contain cytochrome but that it is lost in the latter during preparation. Experiments were alsc conducted on the distribution of cytochrome oxidase. As would be expected this distribution was identical with that found for succinoxidase.

TABLE 2.

Distribution and activity of succinoxidase in different fractions and the presence of cytochrome C.

	Whole homogenate		P granules			M granules			S granules			Supernatant			
Expt.	1	3	1	2	3	1	2	3	1	2	3	1	2	3	Recovery
1	100	13·5	48	66	34	14	21	95	0	0	0	10	13	1	73
2	100	11	37	63	25	14	24	119	2	4	8	4	7	0·5	59
3	100	10	70	74	47	18	19	130	1	1	1	5	5	0·5	94
Mean	100	11·5	52	68	35	16	21	115	2	3	2	6	8	0·7	75
Cytochrome *c.*	+		+			—			—			—			

Column 1 refers to the p.c. of total initial activity recovered in the various fractions; column 2 the p.c. of total recovered activity present in the different fractions and column 3 the activity (μl. O_2/hour) per mg. N.

The figures quoted are derived from the differences in oxygen uptake between flasks without and with added succinate (M/40). Each flask contained 0·5 ml. enzyme (0·2–1·5 mg. N), 0·2 ml. M/5 phosphate (pH 7·5) and cytochrome (2×10^{-5} M) in a final volume of 0·9 ml.

The low percentage recoveries are considered due to the succinate oxidation proceeding further than fumarate in the whole homogenate while in the washed granules the oxidation chain is disrupted.

Other tricarboxylic acid cycle enzymes.

Succinoxidase is a relatively stable enzyme of the cycle. The other enzymes are more labile and their coenzyme needs are in general unknown in the present material. Having determined the succinoxidase partition, however, valid determinations of the distribution of the other enzymes are possible by other means.

Two tubes of whole homogenate were centrifuged at 5,000 × *g* for 5 minutes. From one a sample of supernatant was taken; the other was shaken again to reconstitute the homogenate and a sample taken. This centrifugal procedure removes P granules and some M granules. The residual enzyme, if similar in proportion to succinoxidase, is presumed to be on the remaining M granules.

These studies, as shown in Table 3, indicate that the enzymes of the cycle behave as a complex in the centrifugal field and show that the succinoxidase distribution is typical also for the other enzymes.

TABLE 3.

Distribution of tricarboxylic acid cycle enzymes.

Addition	P.c. stimulation in whole homogenate		P.c. activity removed with centrifugation	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Succinate	104	230	86	91
Citrate	45	82	90	88
α ketoglutarate	48	65	90	90
Fumarate	67	57	86	92
Malate	52	50	87	87
Pyruvate	30	50	90	98
Glutamate	60	76	84	92
Glutamate + malate	130	160	85	89
Endogenous	—	—	81	85

Each flask contained 0·5 ml. enzyme, 0·2 ml. M/5 phosphate (pH 7·5), 2 × 10–5 M cytochrome in a final volume of 0·9 ml. Substrate concentration M/100. The duration of the experiments was 60 minutes during which the whole homogenate control took up 25 and 44 μl. O_2 in the two experiments.

Non-respiratory enzymes.

The distribution of acid phosphatase, apyrase, amylase, and lipase was studied by similar means, except that three supernatants were examined. The results are shown in Table 4. It will be seen that a considerable fraction of these enzymes is present in the ground cytoplasm. The possibility of adsorption of enzyme by the granules after cytolysis must be considered, especially in relation to alkaline phosphatase (cf. Cleland, 1950c). However, assays of the supernatants before washing and after many washings of the precipitate revealed no significant differences in activity.

TABLE 4.

Distribution of some hydrolytic enzymes.

Enzyme	Whole homogenate		Supernatant I		Supernatant II		Supernatant III	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Amylase	100	100	97	95	99	100	103	98
Lipase	100	100	70	73	63	70	57	66
Acid phosphatase	100	100	57	59	50	53	45	49
Apyrase	100	100	65	70	60	66	56	64

The figures refer to the percentage activity remaining in the supernatant after the three successive centrifugations described in Methods. The first removes P granules and some M granules, the second M granules and the third S granules.

The distribution of the glycolytic system and anaerobic pyruvate utilizing system was discussed previously (Cleland, 1950b); the former was, as far as could be judged, entirely soluble and the latter was associated with the granular fraction, the P granules accounting for most of the activity. The distribution of alkaline phosphatase and its relation to the histochemical reaction has also been discussed elsewhere (Cleland, 1950c).

DISCUSSION.

Some questions of cytological terminology may be raised in connection with the present work. Names given to granules on purely morphological grounds would appear to be no more than labels and in recognition of this fact, code designation would seem preferable to the classical terms which have become associated with considerable tradition and some meaning.

The L granules which contaminate all the fractions used here were shown to consist of lipide with no detectable protein or enzymatic constituents. They were previously regarded as "lipide yolk" (Cleland, 1947). The Golgi material of vertebrate cells contains phospholipide (Baker, 1944) and probably consists of small granules (electron microscope studies of Porter and Thompson, 1947; Porter *et al.* 1945), its classical appearance being largely artificial (Palade and Claude, 1949). Now that some of the mystery and vitalism associated with the word Golgi apparatus has been dispelled there would be no valid objection to calling these granules Golgi material; their staining reactions are identical with the classical apparatus, but they may have a higher lipide content than usual (centrifugal behaviour cf. Hibbard, 1946). It is not improbable that the Golgi granules contaminate other granule preparations from mammalian cells (most likely the microsomes) and could thus give rise to invalid phospholipide analyses.

The P granules were distinguished from true mitochondria mainly because of their absence in early oocytes; in their staining reactions in sections they behave like mitochondria, a finding not uncommon in small-sized invertebrate yolk granules. There appears to be no reason to abandon this distinction especially since granules have now been identified which can satisfy the dogma of the ubiquity of mitochondria in all cells. On the other hand it is logically difficult to regard these as classical yolk granules in view of their considerable enzymatic activity; they may therefore be considered functionally but not morphologically homologous with mitochondria. Whether the homologous granules of the sea urchin egg (cf. Harvey, 1941) have similar enzymatic activity cannot be decided from the available literature (Crane and Keltch, 1949; Keltch *et al.*, 1949), although the work considered previously is consistent with this possibility.

The possibility that the enzymatic activity of the P granule fraction is due to nucleoli or chromosomes was considered unlikely for a number of reasons. Several authors studying the respiration of the nucleus and germinal

vesicle have found that there is little activity therein (Brachet, 1937; Dounce, 1943); and if nuclear contaminants were responsible for the activity of the P granules in the present case their activity would have to be much greater than the activity of the mitochondria. It is furthermore probable that enzymes are at the surface of granules; the high mass-surface ratio of the nucleoli in comparison with that of the mitochondria suggests that much enzyme activity of the former is improbable.

The M granules conform to some of the criteria associated with the term mitochondria. Granules similar in size occur widely in the tissues of the oyster and, what is more important, these granules are nucleo-protein in nature and have a strong complement of respiratory enzymes. While they have not been demonstrable in early oocytes this may well be due to technical difficulties. They may therefore be considered both functionally and morphologically homologous with mitochondria.

There appears to be no reason why the S granule fraction should not be homologized with the mammalian microsomes.

The composition of the oyster granules is compared with mammalian granules in Table 5.

It will be seen that the oyster granule fractions have a higher nucleic acid content than most of the normal mammalian tissues. There is evidence in the Table that in neoplastic tissues this is also the case. The high nucleic acid content of the ground cytoplasm of the oyster egg is noteworthy and it is interesting to note that Price *et al.* (1949) have found that it is in the ground cytoplasm that much of the relative and absolute increase in nucleic acid content occurs when normal liver becomes neoplastic. It would appear that in tissues rich in ribonucleic acid *both* the granules and ground cytoplasm may show a higher nucleic acid content than in tissues poorer in nucleic acid.

The presence of considerable amounts of alkaline phosphatase in cell granules has been described by Moog and Steinbach (1946) in chick embryos and by Cleland (1950c) in various guinea-pig tissues. Steinbach and Moog (1945) have shown that considerable amounts of apyrase are also found in the particulate fractions of the chick embryo. Meyerhof and Geliazkowa (1947) and Schneider (1946) have shown that a large proportion is bound to rat brain and rat liver particulates. Le Page and Schneider (1949) have investigated the distribution of the glycolytic system in rat liver. The results, while difficult to interpret, are not inconsistent with those found in the oyster material.

Functional dissimilarities between the various granule fractions in cells seem to be well established both by enzyme analysis and by more complex studies of metabolism (e.g. P_{32} turnover; Aida 1949, Jeener 1949). On the other hand, the question of the inconvertibility of the granule groups is much more difficult, if not impossible, to decide.

TABLE 5.

Granule contents and compositions in different species.

Species	Organ	Whole homogenate			P granules			Mitochondria			Microsomes			Supernatant			Reference
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
Rat	Liver	100	100	4·5				27	30	4·8	16	36	9·5	39	22	2·3	Price *et al.* (1950)
Rat	Liver	100	100	4·3				27	7	1·2	19	53	12·3	41	23	2·4	Schneider (1948)
Rat	Liver	100	100	3·6				25	18	2·5	9	28	9·4	31	21	2·5	Hogeboom *et al.* (1948)
Rabbit	Liver	—	—	—				—	—	4·5	—	—	5·1	—	—	—	Aida (1949)
Rabbit	Liver	100	100	2·9				10·6	10	2·7	14	32	6·5	49	32	1·9	Le Page and Schneider (1948)
Mouse	Liver	100	100	7·3				17	12	5·9	18	36	14	45	35	10·4	Barnum and Huesby (1948)
Oyster	Egg	100	100	14·6	30	12	6·8	4·3	2·7	10	3·6	3·3	16	41	56	21	Present paper
Rat	Liver	100	100	5·3				22	17·5	4·2	—	—	—	—	—	—	Schneider (1946b)
Rat	Hepatoma	100	100	9				8·6	10·6	11·2	—	—	—	—	—	—	Schneider (1946b)
Rat	Liver	100	100	4·0				28	29	4·2	10·3	23	8·9	40	28	2·8	Price *et al.* (1949)
Rat	Hepatoma	100	100	5·8				10·6	9·8	5·5	10	17	9·8	43	43	5·9	
Rat	Flexner Jobling carcinoma	100	100	5·7				8	10	7·5	9	17	11·5	50	32	2·7	Le Page and Schneider (1948)

The figure in column 1 is the p.c. of total protein (or total N), in 2 the p.c. of total ribonucleic acid (from pentose or phosphorus) and 3 the p.c. of nucleic acid in the protein contained in the fraction (calculated from fat free dry weight or total N). The nuclear fraction has been omitted from the Table.

From the chemical side it is not difficult to picture how a nucleoprotein micelle of the ground cytoplasm could, either by its own synthetic activity or by assimilation of formed molecules from the surrounding cytoplasm, yield a microsome with a higher protein and lipide content, but without any significant accession or production of enzymes. The steps from here to mitochondria and, in the case of the oyster, to P granules are not difficult logical steps; they require merely an accession of more protein, some of which is enzymatically active. The only positive evidence suggesting such steps appears to be in the suggestion of Barnum and Huesby (1948) that a continuous spectrum of granules exists in cytoplasm. Observations on living centrifuged cells, which are the only satisfactory control of granule fractionations, by reason of the usually sharp boundaries, suggest, however, that granular categories have few connecting links. In view of this, the possibility must be entertained that the continuous granule spectrum is due to fragmentation. On the other hand, if the microsomes and mitochondria represented stable configurations of granule and the development of the larger granules was saltatory, no obvious connecting link would be expected. The rapid appearance of various granular categories, e.g. secretory granules (cf. Bowen, 1929) and the small protein yolk granules of invertebrate eggs suggests that the above alternative is not unlikely.

The evidence from cytological studies of granule origins is very subjective. Similar staining reactions of various granules can be regarded as no more than suggestive even when the mechanism of the staining reaction is understood. Mere spatial contiguity of granules seems also of little value especially in cases where lipide granules such as the Golgi apparatus are observed in relation to other granule types. Examples of the derivations of various granular categories during invertebrate oogenesis are reviewed by MacBride and Hewer (1931); the more recent literature follows the same lines. Considerable difference of opinion is evident when different workers study the same form and the derivations in different forms by the same author tend to be similar; trends are distinguishable in different schools and different time periods. The wide differences of opinion, together with the above comments, suggest that work like this is of limited value.

The more detailed and precise enzymatic and analytical studies described in the present paper have not advanced the solution of this problem of granule origins much further than the purely morphological approach. They have, however, indicated similarities of composition and thus made the granule progression more credible. The most satisfactory approach to the problem would be by the production of centrifugal merogons. Harvey (1946) has already reported the *de novo* formation of mitochondria in sea urchin embryos derived from the fertilized clear quarter of the egg. Even here the possibility that some mitochondria have been trapped in the cortical gel is difficult to exclude.

SUMMARY.

Methods are described for the centrifugal separation of the following granules from oyster egg homogenates: P granules (protein yolk), M granules (mitochondria), S granules (microsomes), and L granules (lipide yolk or Golgi apparatus).

With the exception of the latter, which are pure lipide, these granules show an increasing nucleic acid content with decreasing size, the ground cytoplasm containing the highest nucleic acid concentration.

Succinoxidase and a number of other enzymes of the tricarboxylic acid cycle are present on both P and M granules but the former is about four times as active per unit of total nitrogen in the M granules. No significant tricarboxylic acid cycle activity is demonstrable in the S granules or the ground cytoplasm.

Amylase is confined to the ground cytoplasm; lipase, acid phosphatase and apyrase are distributed approximately equally between ground cytoplasm and granules.

Acknowledgments. This work has been aided by a grant from the University of Sydney Cancer Research Fund. The oysters used were a gift from the C.S.I.R.O. Fisheries Division, Cronulla.

REFERENCES.

Aida, G. L. (1949): Biochem. J., 45, p. 422.
Baker, J. R. (1944): Quart. J. mic. Sci., 85, p. 1.
Ballentine, R. (1940): J. cell. comp. Physiol., 16, p. 39.
Barnum, C. P. and Huesby, R. A. (1948): Arch. Biochem., 19, p. 17.
Bensley, R. R. and Hoerr, N. L., (1934): Anat. Rec., 60, p. 449.
Bowen, R. (1929): Quart. Rev. Biol., 4, p. 299, 484.
Brachet, J. (1937): Science, 86, p. 225.
Chantrenne, H. (1944): Enzymologia, II, p. 213. (C.A. 40: 2482.)
Cleland, K. W. (1947): Proc. Linn. Soc. N.S.W., 72, p. 159.
Cleland, K. W. (1950a): *Ibid.*, in press.
Cleland, K. W. (1950b): *Ibid.*, in press.
Cleland, K. W. (1950c): *Ibid.*, 75, p. 54.
Cowdry, E. V. (1918): Carnegie Pubs., 272, p. 39.
Crane, R. K. and Keltch, A. K. (1949): J. gen. Physiol., 32, p. 503.
Dounce, A. L. (1943): J. biol. Chem., 147, p. 685.
Du Buy, H. G. *et al.* (1949): J. Nat. Cancer Inst., 9, p. 325.
Harvey, E. B. (1941): Biol. Bull., 81, p. 114.
Harvey, E. B. (1946): J. exp. Zool., 102, p. 253.
Harvey, E. B. and Lavin, G. I. (1944): Biol. Bull., 86, p. 163.
Hibbard, H. (1946): Quart. Rev. Biol., 20, p. 1.
Hogeboom, G. H. *et al.* (1946): J. biol. Chem., 165, p. 615.
Hogeboom, G. H. *et al.* (1948): *Ibid.*, 172, p. 619.
Hutchens, J. O. *et al.* (1942): J. cell. comp. Physiol., 20, p. 113.
Jeener, R. (1949): Nature, 163, p. 837.

Joyet Lavergne, P. (1927–38): Papers in C.R. Soc. Biol., C.R. Acad. Sci. and Protoplasma.
Keltch, A. K. *et al.* (1949): Biol. Bull., 97, p. 242.
Kennedy, E. P. and Lehninger, A. L. (1949): J. biol. Chem., 179, p. 959.
Kingsbury, B. F. (1912): Anat. Rec., 6, p. 39.
Lazarow, A. (1943): Biol. Symp., 10, p. 9.
Lazarow, A. and Barron, E. S. G. (1941): Anat. Rec., 79, p. 41 (Supp.).
Le Page, G. A. and Schneider, W. C. (1948): J. biol. Chem., 176, p. 1,028.
MacBride, E. W. and Hewer, H. R. (1931): Zoology (Section III) in Piney A. (Ed) "Recent Advances in Microscopy," Churchill, London.
McRary, W. L. and Slattery, M. C. (1945): Arch. Biochem., 6, p. 151.
Mayer, A. *et al.* (1914): J. Physiol. Path. gen., 16, p. 607.
Meyerhof, A. and Geliazkowa, N. (1947): Arch. Biochem., 12, p. 405.
Moog, F. and Steinbach, H. B. (1946): J. cell. comp. Physiol., 28, p. 209.
Navez, H. E. and Harvey, E. B. (1935): Biol. Bull., 69, p. 342.
Palade, G. E. and Claude, A. (1949): J. Morph., 85, p. 71.
Porter, K. R. *et al.* (1945): J. exp. Med., 81, p. 233.
Porter, K. R. and Thompson, H. P. (1947): Cancer Res., 7, p. 341.
Price, J. M. *et al.* (1949): *Ibid.*, 9, p. 96.
Price, J. M. *et al.* (1950): *Ibid.*, 10, p. 19.
Recknagel, R. O. (1950): J. cell. comp. Physiol., 35, p. III.
Schneider, W. C. (1946a): J. biol. Chem., 165, p. 585.
Schneider, W. C. (1946b): Cancer Res., 6, p. 685.
Schneider, W. C. (1948): J. biol. Chem., 176, p. 259.
Shapiro, H. (1935): J. cell. comp. Physiol., 6, p. 101.
Steinbach, H. B. and Moog, F. (1945): *Ibid.*, 26, p. 175.
Warburg, O. (1914): Arch. Physiol., 158, p. 189.

A SIMPLE APPARATUS FOR DRYING SMALL SPECIMENS OF SERA

by HENRY FRANCIS HOLDEN[1] AND JEAN SCOTT

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(Accepted for publication 1st August, 1950.)

In institutes of biological research it is often desirable to maintain stocks of many small specimens of dried sera or other materials for use at later dates. Since instruments for drying sera from the frozen state available commercially are large and expensive, with other features that did not attract us, it was decided to build one as economically as possible that should dry up to fifty small specimens at a time. Such an instrument would well serve a small group of workers without requiring elaborate skill or any very deterrent expense.

The apparatus, which is mounted on a small wheeled table, comprises a small rotary pump, a vertical manifold with fifty-two side-tubes, a condenser cooled with solid carbon dioxide in a one-gallon vacuum jar, and a Pirani vacuum gauge. The table has a top 60 cm. × 55 cm., 72 cm. above the ground and a lower shelf for the pump 23 cm. above the ground. It was made as small as possible with a view to its use in a confined space. A slightly larger one with fittings to hold the ancillary equipment might better suit some users. The rotary pump has a maximum pumping speed of about 0·5 litre per sec. and an ultimate vacuum of 3–5 microns. It is mounted on rubber sponge on the lower shelf of the table. The manifold and condenser are made of pyrex glass and connected with a B.29 ground joint. Glass was chosen because it could be readily worked by one of us (H.F.H.), into a vacuum-tight unit. The glass condenser is ample for the small amount of water condensed in a run. Glass has the advantage, too, that water does not condense on the external surface of the manifold. The condenser is additionally secured to the manifold with light steel springs. The manifold is so mounted on a stand that it can be moved only in a vertical direction, and then turned and locked only when the condenser is clear of the vacuum jar. The condenser can then be detached for emptying, and the vacuum jar removed if necessary.

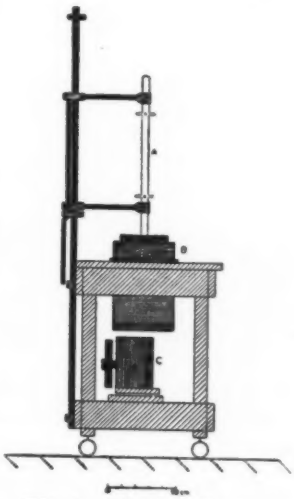

Fig. 1. Diagram of apparatus. Drawn to scale. A, Manifold. B, Vacuum flask. C, Pump.

Two vacuum jars are used. They are standard commercial "refills" of pyrex-glass, mounted in rectangular wooden boxes with bakelite rims. Around the top of each box is screwed a wooden ledge to permit of its being lowered into, but not through, a hole in the top of the table.

[1] This work was supported by the Australian National Health and Medical Research Council.

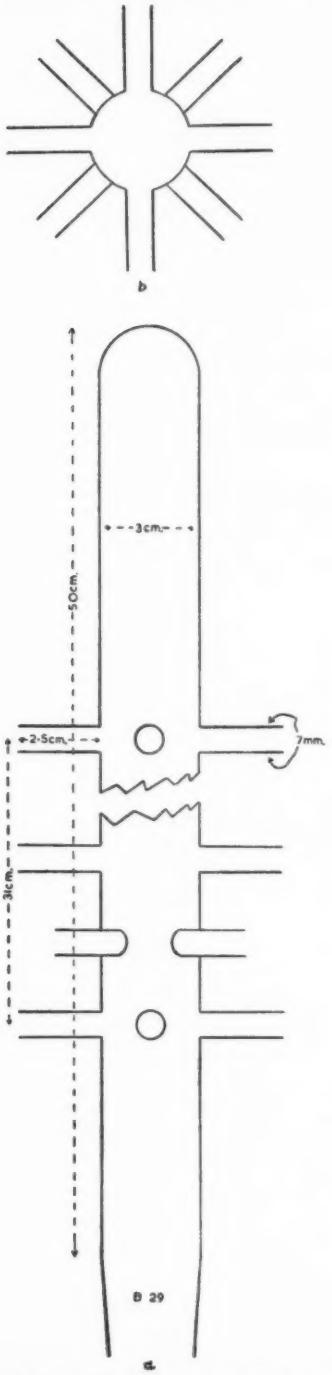

Fig. 2. a, Manifold. b, Cross section plan of manifold.

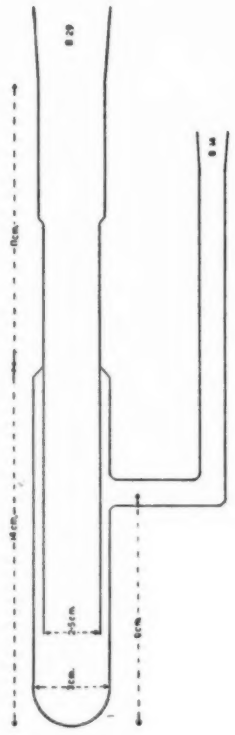

Fig. 3.

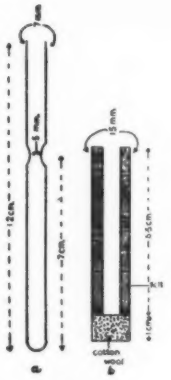

Fig. 4.

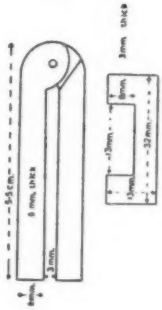

Fig. 5.

Fig. 3. Condenser.
Fig. 4. a, Pyrex glass ampoule. b, Test tube.
Fig. 5. Clamp.

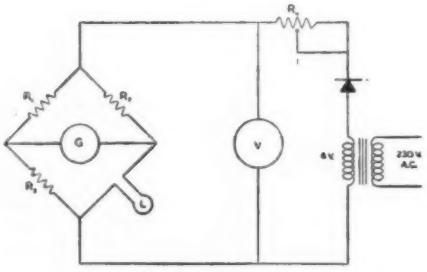

Fig. 6. Electrical circuit of Pirani gauge.

$R_1 = R_2 =$ 50 ohms.
$R_3 =$ 100 ohms.
$R_4 =$ 350 ohms.
L = filament of 60 w. lamp.
G = 0–1 milliammeter.
V = 0–6 v. voltmeter.

Additional equipment comprises: a set of glass plugs, tapered to facilitate insertion, to fit the rubber tubes on the side tubes of the manifold; a brass clamp for clamping each rubber tube, while the glass plug is being removed and the ampoule inserted; test tubes lined with felt and cotton wool, which are slipped over the ampoules as soon as they are attached to the manifold, thus preventing the specimens from thawing before drying; six small cans with loose fitting lids in which the sera are frozen in the ampoules; an insulated wooden box in which the test tubes are cooled, and a small ring burner for sealing the ampoules.

A costly commercial Pirani gauge is not required. One was made very simply using two meters to eliminate the two-way switch which can cause trouble. Small meters supplied by wireless dealers have proved adequate. The three fixed resistors are of copper-nickel alloy size 40 B and S., wound on one flat strip of bakelite. The rectifier is of the 18 volt, 0·15 amp. size. The transformer is a car type wireless transformer, operated in reverse. It delivers at 6 v. with 230 v. applied to the high tension winding. The thermo-sensitive element was made as follows: The solder at the base of a 60 w. lamp was melted and tapped out; the base cap was removed by boiling for one hour in 10 p.c. sodium hydroxide solution, the lamp was opened where it had been sealed off; the glass bulb was cut away, leaving the filament supported on the "pinch." A small brass case with vacuum-tight closure at the ends and a B.12 standard taper joint was made, and the lamp filament secured inside it, with the wires soldered to vacuum-tight insulated terminals in the base of the case. Despite the obvious theoretical errors in this device, it amply suffices for the purpose in view. Its justification is that the total cost of the equipment has been maintained at a level which renders the technique the more widely available.

These additional suggestions may be helpful.

The pyrex ampoules will not hold for drying more than 0·25 ml. in each. For larger volumes small bulbs must be made. Ampoules are made in pairs, heating the middle of the tube to form the bottoms of two ampoules. This diminishes the danger of a minute capillary leak at the base. The mouth must be flame-polished, and the constriction as slight as is consistent with reasonable ease of sealing. Ampoules can be marked with a solution of shellac in absolute alcohol, filtered and allowed to evaporate till it appears slightly viscous and coloured with methylene blue. Commercial quick-drying glass inks are available. A piece of pressure-adhesive transparent tape round the ampoule protects the marks.

The glass plugs may be very lightly greased with a trace of vaseline. Either vaseline or a rubber-vaseline stopcock grease may be used on the ground joints. Costly high-vacuum lubricants are unnecessary here. The glass plugs are inserted in the rubber tubes shortly before the apparatus is used. If they remain in position longer than necessary the rubber becomes permanently set.

The operation of drying sera is performed as follows: In the morning the specimens are measured into the numbered sterile ampoules with graduated sterile Pasteur pipettes, care being taken to avoid bubbles or the lodgement of liquid in the constriction. The ampoules are placed in the cans and frozen in a vacuum jar under crushed solid carbon dioxide; a liquid is not necessary. At about 3 p.m. the condenser is connected to the manifold and the apparatus lowered into the second vacuum jar on the table. This jar is two-thirds filled with solid carbon dioxide and a layer of cotton-wool put on top. The test tubes are put to cool upside down under solid carbon dioxide in their insulated box. They are so placed that they may be quickly withdrawn with the gloved hand. The glass plugs are put in the rubber tubes. At 4 p.m. the pump is started and the vacuum tested with the gauge. Then, one at a time, starting

on the lowest tier, each tube is clamped, the glass plug withdrawn, the wool plug removed from the ampoule and the ampoule inserted in the rubber tube. The clamp is removed and the test-tube at once slipped on the ampoule. A helper to perform this last action is useful but not essential. The fifty-two ampoules of this apparatus can be comfortably dealt with by one person in well under an hour. After a final inspection of the pressure and a packing of the solid carbon dioxide in the jar the apparatus is left to run overnight. Next morning the test-tubes are returned to their box and the ampoules sealed off under vacuum, starting from the top. Soon after sealing is completed the scraps of glass tube are removed from the rubber tubes and also the glass plugs from any unoccupied tubes. The manifold and condenser may be left in position to allow the apparatus to reach the temperature of the room at leisure or they may be raised to prepare for another run if the jar is kept cold.

From the technical aspect this equipment has the following advantages:

It is simple; it can be easily set up and left running unattended.

The ampoules are sealed without exposure to the air of the room as with some large-scale machines.

It is flexible. For special purposes a different manifold or condenser can be used.

It can be readily made by any reasonable laboratory technician. The materials cost about £25 in addition to £50 for the vacuum pump.

SUMMARY.

For drying at a time 50 specimens of sera, each of 0·25 ml., an inexpensive apparatus is used. It comprises a glass manifold with 50 side tubes, a glass condensor cooled with solid CO_2, a vacuum jar and a small rotary pump. To prevent the sera from thawing before they are dried the ampoules are enclosed in jackets.

SPECIFIC SERUM AGGLUTINATION OF SHEEP ERYTHROCYTES SENSITIZED WITH BACTERIAL POLYSACCHARIDES

by LILA HAYES

(From the Institute of Epidemiology and Preventive Medicine, Prince Henry Hospital, Sydney).

(Accepted for publication 26th September, 1950.)

Erythrocytes are capable of adsorbing numerous substances and thereby becoming specifically agglutinable by serum antibody directed against the adsorbed substance. Haemagglutination by specific antisera of erythrocytes sensitized with bacterial fractions has been observed by Keogh, North and Warburton (1947, 1948), Middlebrook and Dubos (1948), Warburton, Keogh and Williams (1949), Hayes and Stanley (1950) and Alexander, Wright and Baldwin (1950). The present paper describes preliminary observations on haemagglutination of sheep erythrocytes sensitized with polysaccharides isolated from one strain only of each of the following organisms: *Bact. coli, Shigella sonnei, C. diphtheriae* (mitis), *Staph. aureus, Staph. albus* and *Str. pneumoniae* (type 111). Antisera have been prepared against both the intact organisms and the corresponding fraction. Agglutination and precipitation, as well as haemagglutination tests, have been performed in an attempt to compare the results obtained from these different serological tests involving specific bacterial polysaccharides.

Materials and Methods.

Preparation of bacterial polysaccharides. The method described by Hayes and Stanley (1950) was used for the preparation of all fractions. The cells of *Bact. coli* were disrupted mechanically, those of *Shigella sonnei, C. diphtheriae, Staph. aureus* and *Staph. albus* were disrupted by glycine (Maculla and Cowles, 1948) and those of *Str. pneumoniae* by 1 p.c. bile. *Shigella sonnei* growth was harvested after 6 hours' incubation at 37° C. and the remaining strains after 24 hours' incubation. Five volumes of ethanol were used for precipitating polysaccharides from *C. diphtheriae, Staph. aureus* and *Staph. albus.*

Preparation of specific anti-sera. Heat-killed vaccines of *Bact. coli, Shigella sonnei, Staph. aureus* and *Str. pneumoniae* were prepared from smooth strains. Rabbits were injected intravenously with four increasing doses of vaccine, bi-weekly, were rested for one week and then bled (vaccine antisera, V). *C. diphtheriae* vaccine was prepared and administered according to the method of Hewitt (1947).

Polysaccharide antisera (P) were prepared by intravenous injection of rabbits with six increasing doses of polysaccharide at 2 day intervals; the animals were rested for one week and then bled. The initial immunizing dose of each polysaccharide was determined by the toxicity of the fraction for rabbits.

Preparation of washed sheep erythrocytes. Fresh defibrinated sheep blood was centrifuged, the packed cells washed three times in saline (0·9 p.c. sodium chloride) and stored at 4° C.

The packed erythrocytes were not used for haemagglutination or haemolysis tests if they were more than 5 days old or if they showed excessive haemolysis before that time.

Sheep erythrocytes treated with borax (Rappaport and Skariton, 1949) were satisfactory for haemagglutination tests after six weeks' storage at 0° C.

Sensitization of sheep erythrocytes with polysaccharides. Sufficient packed erythrocytes to give approximately a 6 p.c. suspension were added to any desired volume of saline containing polysaccharide of known concentration. The suspension was incubated at 37° C. for 1 hour and then centrifuged; the packed cells were washed three times with saline. The sensitized washed erythrocytes were diluted to approximately 1 p.c. for haemagglutination and haemolysis tests. The concentration of polysaccharide used for sensitizing the erythrocytes in any given experiment is referred to as the sensitizing dose of polysaccharide (S.D.P.), and is expressed as the reciprocal of the dilution used.

Haemagglutination test. Sheep erythrocytes were treated with the desired sensitizing dose of any particular polysaccharide. Serial twofold dilutions of the antisera to be tested were made in saline (1 ml. volumes), in 6 in. × ½ in. round-bottomed test tubes; the control tubes contained (1) no serum and (2) normal rabbit serum 1:20. One ml. of 1 p.c. sensitized erythrocytes was added to each tube and the tubes were well shaken. Final readings were made after the tubes had stood 18 hours at room temperature. In the control tubes and tubes showing negative readings the erythrocytes settled out as a small, central, sharply demarcated disc which slipped from position when the tube was tilted. In strongly positive reactions the erythrocytes settled out as a broad disc with a serrated margin, covering the bottom of the tube. The haemagglutination titre of any serum sample was the *final* dilution contained in the last tube where the erythrocytes had settled in a disc which was broader than that of the control tube, showed a fine aggregation of cells at the margin and would not shift from position when the tube was tilted.

Haemolysis test. Fresh guinea-pig serum (pooled from three animals) was used as a source of complement. Before each test the complement was titrated with normal and sensitized sheep erythrocytes to detect the presence of normal lysins for sheep erythrocytes. The lowest dilution of complement which showed no haemolysis was selected for use. Because of the preliminary nature of the haemolytic tests it was considered unnecessary to standardize the complement used in each test, in terms of minimum haemolytic doses for normal sheep erythrocytes; as a result, small differences in haemolytic titres of antisera were observed with different batches of complement. Serial twofold dilutions of the sera to be tested were made in the desired concentration of complement (1 ml. volumes); the control tubes contained no serum. One ml. of 1 p.c. suspension of sensitized erythrocytes was added to each tube. The tubes were well shaken and put in a waterbath at 37° C. for 1 hour when the final reading was taken. The haemolytic titre of the serum was the *final* dilution in the last tube showing *complete* haemolysis. Partial haemolysis occurred in high dilutions of antisera and no clear cut end point was visible in such tubes. The haemolytic titre of specific antisera for normal sheep erythrocytes was determined before each haemolytic test was performed.

Agglutination and precipitation tests. Heat-killed suspensions of *Bact. coli, Shigella sonnei Staph. albus, Staph. aureus* and *Str. pneumoniae* were used in agglutination tests. *C. diphtheriae* agglutinating suspension was prepared by the method of Hewitt (1947). For the precipitation tests polysaccharide solutions of desired concentration were prepared in saline. All agglutination and precipitation tests were carried out in a precipitin bath (modification of the model described by Platt, 1941), which was so constructed that it was not necessary to remove the tubes to take a reading. Final readings were made for both agglutination and precipitation tests after the tubes had been in the precipitin bath at 55° C. and 37° C. respectively, for 18 hours. The agglutination or precipitation titre of a serum sample was the *final* dilution in the last tube, when examined with a × 2 hand lens, showing agglutination or precipitation.

Chemical tests. The tests described by Hayes and Stanley (1950) were used for chemical examination of the bacterial fractions.

EXPERIMENTAL.

Sensitization of sheep Erythrocytes for different periods of time.

In the experiments described below, sheep erythrocytes were sensitized for 1 hour at 37° C. Before selecting this time a preliminary experiment using *Shigella sonnei* polysaccharide was conducted to determine (1) if the length of time used for sensitizing the erythrocytes with any given concentration of polysaccharide affected the haemagglutination titre of the antiserum as read after 18 hours at room temperature, and (2) to observe the occurrence of an optimum serum dilution in the haemagglutination system. Erythrocytes were sensitized with *Shigella sonnei* polysaccharide (1:10,000) at 37° C. for 5, 10, 15, 30, 45, 60, 90 and 120 minute periods, were washed three times with saline and diluted to 1 p.c. suspensions. Haemagglutination tests using vaccine antiserum were set up and the tubes observed closely. The dilution of antiserum in the first tube showing haemagglutination and the time taken were recorded. The final haemagglutination titre of the antiserum was recorded after 18 hours. From Table 1 it can be seen (1) that the shorter the sensitizing time the longer the time required for haemagglutination to occur in the first tube, (2) that an optimum serum dilution was exhibited in all cases, haemagglutination occurring at dilutions of antiserum 1:80 and 1:160 first and later at 1:20 and, (3) the haemagglutination titre of the antiserum, as read at 18 hours, was not affected if a sensitizing time of 30 minutes or longer was employed. Sensitization for 1 hour was selected as an optimum time for routine haemagglutination tests.

TABLE 1.

Haemagglutination tests using sheep erythrocytes sensitized for different periods of time.

(Shigella sonnei *polysaccharide, 1:10,000, and vaccine antiserum.*)

Sensitizing time (mins.)	First tube showing haemagglutination		Titre of antiserum at 18 hours
	Time (mins.)	Dilution antiserum	
5	18	80	20,480
10	16	80	20,480
15	8	80	20,480
30	6	80	40,960
45	5	80	40,960
60	3	80 and 160	40,960
90	3	80 and 160	40,960
120	3	80 and 160	40,960

The effect of decreasing sensitizing doses of polysaccharide and increasing sensitizing times on the haemagglutination titres of specific antisera.

Sheep erythrocytes were sensitized for 1 hour at 37° C. with decreasing concentrations of polysaccharides isolated from *Bact. coli, Shigella sonnei, C. diphtheriae, Staph. aureus, Staph. albus* and *Str. pneumoniae.* Haemagglutination titres of both specific vaccine and polysaccharide antisera (Table 2) show, with the exception of *C. diphtheriae* and *Staph. aureus,* that in each case the vaccine antiserum was more active than the corresponding polysaccharide antiserum and that the haemagglutination titre of each antiserum falls as the concentration of polysaccharide in the sensitizing dose decreases.

In a further experiment erythrocytes were sensitized with decreasing concentrations of *Bact. coli* polysaccharide for 24 hours, the first 6 hours at 37° C. and the remainder of the

time at 4° C. Haemagglutination tests using *Bact. coli* vaccine antiserum were performed when the erythrocytes had been sensitized for 1, 3½, 6 and 24 hours. From Table 3 it can be seen that the same haemagglutination titre (1:40,960) of the specific antiserum could be demonstrated when the erythrocytes were (1) sensitized with a strong polysaccharide solution (1:10,000) for 1 hour and (2) with a weaker polysaccharide solution (1:320,000) for 6 hours. Prolonged sensitization of erythrocytes with any particular polysaccharide concentration did not increase the haemagglutination titre of the antiserum above 1:40,960. Similar results were obtained when the above experiment was performed using *Shigella sonnei* and *Staph. aureus* polysaccharides and specific antisera.

TABLE 2.

Haemagglutination titres of antisera using decreasing concentrations of polysaccharide for sensitizing sheep erythrocytes.

Polysaccharide	Antisera	Sensitizing dose of polysaccharide						
		5,000	10,000	20,000	40,000	80,000	160,000	320,000
Bact. coli	V	—	40,960	20,480	20,480	10,240	5,120	1,280
	P	—	5,120	5,120	2,560	1,280	640	<20
Shigella sonnei	V	—	40,960	10,240	5,120	2,560	1,280	<20
	P	—	10,240	5,120	2,560	2,560	1,280	<20
C. diphtheriae	V	640	320	160	80	40	<20	—
	P	1,280	320	320	80	40	20	—
Staph. aureus	V	640	640	320	320	320	160	—
	P	5,120	2,560	2,560	2,560	2,560	1,280	—
Staph. albus	V	1,280	640	320	20	20	<20	—
	P	nil						
Str. pneumoniae	V	—	1,280	1,280	1,280	640	320	160
	P	—	nil					

— not done.

TABLE 3.

Haemagglutination titres of Bact. coli *vaccine antiserum using sheep erythrocytes sensitized for increasing periods of time with decreasing amounts of* Bact. coli *polysaccharide.*

Sensitizing time (hours)	Sensitizing dose of polysaccharide					
	10,000	20,000	40,000	80,000	160,000	320,000
1	40,960	20,480	20,480	10,240	5,120	5,120
3½	40,960	40,960	20,480	20,480	20,480	10,240
6	40,960	40,960	40,960	40,960	40,960	40,960
24	40,960	40,960	40,960	40,960	40,960	40,960

Multiple sensitization of sheep erythrocytes.

(a) Sheep erythrocytes were sensitized "in series" with polysaccharides of *Bact. coli* (1: 10,000), *Shigella sonnei* (1: 10,000), *Staph. aureus* (1: 5,000) and *C. diphtheriae* (1: 5,000), in the above order. Sufficient washed packed erythrocytes to give approximately a 6 p.c. suspension were added to 8 ml. of the first polysaccharide, sensitized at 37° C. for 1 hour

and then washed 3 times with saline. Eight ml. of the second polysaccharide were then added and the procedure repeated until the erythrocytes had been treated "in series" with all four polysaccharides.

(b) Sheep erythrocytes were also sensitized with a solution containing a mixture of the above polysaccharides. The concentration of each polysaccharide in the mixture was the same as that used in the "series" experiment. Sufficient erythrocytes to give approximately a 6 p.c. suspension were added to the polysaccharide mixture, sensitized at 37° C. for 1 hour, washed 3 times in saline and resuspended in saline for use.

The usual haemagglutination tests were then set up using sheep erythrocytes sensitized with the polysaccharides in series, in mixed solution and individually (controls). The results of the haemagglutination tests shown in Table 4 indicated that there was no interference with any specific haemagglutination system due to the treatment of the erythrocytes with more than one polysaccharide.

TABLE 4.

Haemagglutination and haemolytic titres of antisera using multiple-sensitized sheep erythrocytes.

Polysaccharide	S.D.P.	Antisera	Test*	Control	Mixture	Series
Bact. coli	10,000	V	H	40,960	40,960	40,960
			L	163,940	163,940	163,940
Shigella sonnei	10,000	V	H	40,960	40,960	40,960
			L	81,920	81,920	81,920
Staph. aureus	5,000	P	H	2,560	1,280	2,560
			L	10,240	10,240	20,480
C. diphtheriae	5,000	P	H	1,280	1,280	640
			L	10,240	10,240	20,480

* H = haemagglutination test. L = haemolysis test.

The addition of complement to the haemagglutination system (haemolysis test).

Fisher and Keogh (1950) have reported lysis by complement of erythrocytes which have adsorbed a bacterial component and its antibody. Sheep erythrocytes sensitized with polysaccharides from *Bact. coli, Shigella sonnei, C. diphtheriae, Staph. aureus, Staph. albus* and *Str. pneumoniae* were readily lysed in the presence of specific antibody and complement (Table 5). The rabbit antisera prepared against specific organisms and the corresponding polysaccharides showed the presence of lysins for normal sheep erythrocytes to a titre of 1 : 30 or less. This in no way interfered with the results as the lowest dilution of antiserum used in the haemolysis tests was always greater than 1:80. Complement titrations showed the absence of lysis for sheep erythrocytes in dilutions higher than 1:20. The *final* dilution of complement used in most of the haemolysis experiments was 1:40.

From Table 5 it can be seen that (1) the haemolytic titres of specific antisera were equal to or higher than the corresponding haemagglutination titres and (2) as in the haemagglutination tests, with the exception of *Staph. aureus* and *C. diphtheriae*, higher titres in the haemolytic tests were obtained with vaccine than with polysaccharide antisera. The haemolysis test is more rapid than the haemagglutination test (1 hour at 37° C. as opposed to 18 hours at room temperature) and appears to be more sensitive.

TABLE 5.

Haemagglutination and haemolytic titres of antisera using sheep erythrocytes sensitized with potassium periodate-treated polysaccharides.

Polysaccharide	S.D.P.	Antisera	Haemagglutination		Haemolysis	
			Control	KIO_4	Control	KIO_4
Bact. coli	10,000	V	40,960	20	163,840	5,120
		P	5,120	20	20,480	2,560
Shigella sonnei	10,000	V	40,960	320	163,840	2,560
		P	10,240	20	10,240	1,280
C. diphtheriae	5,000	V	640	640	10,240	10,240
		P	1,280	1,280	10,240	10,240
Staph. aureus	5,000	V	640	640	2,560	2,560
		P	5,120	5,120	10,240	10,240
Staph. albus	5,000	V	640	640	640	640
		P	Nil	Nil	Nil	Nil
Str. pneumoniae	5,000	V	1,280	1,280	40,960	20,480
		P	Nil	Nil	Nil	Nil

Experiments using erythrocytes sensitized with *Bact. coli, Shigella sonnei, Staph. aureus* and *C. diphtheriae* polysaccharides show that the haemolytic titre of a specific antiserum falls as the sensitizing dose of polysaccharide is decreased (see Table 6), and as the concentration of complement is decreased (see Table 7). When small amounts of complement were used prozones were observed in the different haemolytic systems. In the case of *Bact. coli* and *Shigella sonnei* inhibition of haemolysis occurred in serum dilutions up to 1:160 when complement 1:640 was employed; with *C. diphtheriae* inhibition occurred in serum dilutions up to 1:80 when complement dilutions of 1:320 and 1:640 were used. In experiments using constant concentrations of antiserum it was observed that the stronger the antiserum, the less complement was required to bring about haemolysis of sensitized erythrocytes.

Haemolysis tests performed with multiple-sensitized sheep erythrocytes (as for haemagglutination tests) indicated that treatment of the erythrocytes with more than one polysaccharide in no way interfered with any specific haemolytic system.

TABLE 6.

Haemolytic titres of antisera using decreasing concentrations of polysaccharides for sensitizing sheep erythrocytes.

Polysaccharide	Antisera	Sensitizing dose of polysaccharide						
		5,000	10,000	20,000	40,000	80,000	160,000	320,000
Bact. coli	V	—	163,840	40,960	40,960	40,960	20,480	10,240
	P	—	20,480	5,120	5,120	2,560	1,280	640
Shigella sonnei	V	—	163,840	81,920	40,960	40,960	20,480	10,240
	P	—	10,240	5,120	2,560	2,560	1,280	640
Staph. aureus	V	1,280	640	640	320	320	160	—
	P	10,240	2,560	2,560	2,560	1,280	640	—
C. diphtheriae	V	10,240	5,120	5,120	2,560	2,560	1,280	—
	P	10,240	5,120	2,560	1,280	640	320	—

— not done.

TABLE 7.

Haemolytic titres of specific antisera using decreasing concentrations of complement.

Polysaccharide	S.D.P.	Antisera	Final dilution of complement					
			40	80	160	320	640	1,280
Bact. coli	10,000	V	40,960	20,480	10,240	2,560	1,280*	80
Shigella sonnei	10,000	V	40,960	20,480	10,240	5,120	1,280*	80
C. diphtheriae	5,000	P	5,120	1,280	640	320*	160*	80

* Prozone observed.

Haemagglutination and haemolytic titres of specific antisera using erythrocytes sensitized with potassium periodate (KIO_4)-treated polysaccharides.

The oxidizing effect of the periodate radical on carbohydrates in biological materials is well known. Equal parts of a fresh solution of KIO_4 (M/1,000) and polysaccharide (twice the desired final concentration) were mixed and stood at room temperature for 30 minutes. Glycerol-saline (final concentration 0·01 M) was then added to inactivate the residual periodate. Sufficient washed packed sheep erythrocytes to give approximately a 6 p.c. suspension were added to the treated polysaccharide and incubated at 37° C. for 1 hour. The erythrocytes were then washed with two changes of 0·01 M glycerol-saline, once with saline and resuspended in saline. The usual haemagglutination and haemolytic tests were carried out using the KIO_4-treated polysaccharide, and untreated polysaccharide sensitized erythrocytes. From Table 5 it can be seen that when polysaccharides from *Bact. coli* and *Shigella sonnei* are treated with periodate their ability to sensitize erythrocytes to the action of specific haemagglutinin is markedly reduced. This was not the case with polysaccharides from *C. diphtheriae, Str. pneumoniae, Staph. aureus* and *Staph. albus.* Treatment of the polysaccharides with periodate reduced the haemolytic titre of specific antisera only in the cases of *Shigella sonnei* and *Bact. coli*; the reduction in titre was not as great as that in the haemagglutination tests.

The inactivation of *Bact. coli* and *Shigella sonnei* polysaccharides by potassium periodate suggested the treatment of erythrocytes with periodate. Sheep erythrocytes sufficient to give a 20 p.c. suspension were added to potassium periodate in concentrations from M/2,000 to M/100,000, and stood at room temperature for 30 minutes. Glycerol-saline (final concentration 0·01 M) was then added to inactivate the periodate. The erythrocytes were washed twice with 0·01 M glycerol-saline and once with saline. The packed erythrocytes were then sensitized with *Bact. coli* and *Shigella sonnei* polysaccharides and the usual haemagglutination and haemolysis tests performed. Treatment of the erythrocytes with potassium periodate in no way affected the haemagglutination or haemolytic titres of *Bact. coli* and *Shigella sonnei* vaccine and polysaccharide antisera.

Agglutination tests.

With the exception of *C. diphtheriae*, each antigenic polysaccharide produced an antiserum capable of agglutinating a suspension of the organism from which the polysaccharide was prepared (Table 8). The agglutination titre of any polysaccharide antiserum was in each case less than that of the corresponding vaccine antiserum.

Precipitation tests.

The precipitation titres of vaccine and polysaccharide antisera were determined using the specific polysaccharide in constant concentration (Table 8). *Bact. coli* and *Shigella sonnei* vaccine antisera showed higher titres than did the corresponding polysaccharide antisera. The

opposite was the case with *C. diphtheriae* and *Staph. aureus*. The polysaccharides from *Staph. albus* and *Str. pneumoniae* were precipitated by the specific vaccine antisera but not by the polysaccharide antisera.

Precipitation tests using a constant antibody concentration (Table 8) indicated that *Bact. coli* and *Shigella sonnei* vaccine and polysaccharide antisera were equally effective in detecting the presence of small amounts of the specific polysaccharide. *C. diphtheriae* vaccine antiserum, when undiluted gave a feebly positive ring test but failed to precipitate the polysaccharide if diluted 1: 20. *C. diphtheriae* polysaccharide 1: 160,000 was precipitated by the polysaccharide antiserum 1: 20. *Staph. aureus* polysaccharide antiserum 1: 40, precipitated the polysaccharide at a higher dilution than did the vaccine antiserum. *Staph. albus* and *Str. pneumoniae* vaccine antisera precipitated the specific polysaccharide but the polysaccharide antisera did not.

Chemical examination of the fractions.

The chemical facts presented in Table 9 can give only limited information regarding the composition of the fractions described. The presence of a carbohydrate nucleus, some nitrogenous material, sulphur and phosphorous, however, is evident in all fractions. These qualitative tests reveal no marked differences, as expressed by the presence or absence of certain chemical groupings, between the fractions.

TABLE 8.

Agglutination and precipitation tests.

Antigens	Agglutination		Precipitation							
			Titre of antisera: constant antigen			Titre of antigen: constant antibody			Ring test	
	V	P	Antigen	V	P	Antibody	V	P	V	P
Bact. coli	20,480	2,560	80,000	10,240	5,120	40	5,120,000	5,120,000	++	++
Shigella sonnei	5,120	1,280	80,000	1,280	640	40	1,280,000	1,280,000	++	++
C. diphtheriae	640	Nil	20,000	Nil	640	20	<10,000	160,000	±	++
Staph. aureus	10,240	2,560	20,000	640	1,280	40	40,000	640,000	+	++
Staph. albus	10,240	Nil	10,000	640	Nil	40	640,000	Nil	+	Nil
Str. pneumoniae	640	Nil	10,000	80	Nil	20	20,000	Nil	+	Nil

++ strong positive. + positive. ± trace.

TABLE 9.

Chemical examination of fractions.

Source of fraction	Molisch	Bial	Dische	Biuret (Osborne)	Millon	Sakaguchi	Nitrogen	Sulphur	Phosphorus	P.c. reducing sugar
Bact. coli	+++	+	+	+	++	++	+	+	+++	13
Shigella sonnei	+++	+++	+	++	+++	++	++	—	+++	9
C. diphtheriae	+++	+++	++	+	++	++	+	++	++	30
Staph. aureus	+++	++	±	++	++	++	±	++	++	29
Staph. albus	+++	++	+++	++	+	—	+	++	++	17
Str. pneumoniae	+++	+++	++	++	++	++	++	++	++	10

+++ strong positive. ++ positive. + weak positive. ± trace. — negative.

DISCUSSION.

Haemagglutination occurred with appropriate antisera whether the polysaccharides involved were haptens (*Staph. albus* and *Str. pneumoniae*) or antigens (*Bact. coli., Shigella sonnei,* and *C. diphtheriae*). In any haemagglutination system, as with precipitation and bacterial agglutination, an optimum serum dilution for agglutination was observed; complete inhibition of haemagglutination by low serum dilutions was not observed. For any particular antiserum the haemagglutination titre was higher than the precipitation titre. In the case of *C. diphtheriae,* the vaccine antiserum, if diluted, did not precipitate the polysaccharide but caused agglutination of erythrocytes sensitized with polysaccharide. Collodion pellets coated with specific antigens have been used in agglutination tests by Weir (1941) for detection of small amounts of antibody not detected satisfactorily by precipitation tests. The mechanism of the haemagglutination and collodion pellet agglutination tests is probably similar, but the former is more easily performed as no special preparation of reagents is necessary.

The concentration of polysaccharide used for sensitizing erythrocytes and the time the erythrocytes were in contact with the polysaccharide were important factors in determining the haemagglutination titre of a specific antiserum. Prolonged sensitization of erythrocytes with a strong polysaccharide solution did not reveal a higher haemagglutination titre of the specific antiserum. When erythrocytes were sensitized with a weaker solution of polysaccharide, the longer the sensitizing time, the higher was the haemagglutination titre of the specific antiserum. It would seem that in order to demonstrate small amounts of antibody in a specific antiserum there was an optimum amount of polysaccharide adsorbed on to the red cell surface; if insufficient polysaccharide was adsorbed a lower haemagglutination titre was demonstrated. The fact that multiple-sensitization of erythrocytes affected neither the haemagglutination nor the haemolytic titre of any particular system is of interest. It seems likely that the surface area of the erythrocytes is only partially coated by even the strongest polysaccharide solutions used in these experiments, thus allowing the adsorption of further polysaccharides. Four polysaccharides were used in the above experiments; the total number of polysaccharides which erythrocytes could adsorb without interference with the specific haemagglutination or haemolytic systems involved, has not been determined.

From the haemolytic systems studied here, it is seen that the greater the amount of polysaccharide adsorbed on to the erythrocyte surface and the greater the amount of complement present, the smaller was the amount of specific antibody required for haemolysis. When the amount of complement used was relatively small haemolysis was inhibited in low dilutions of antiserum, haemagglutination occurring in the prozone tubes. When constant concentrations of antisera and increasing dilutions of complement were used in haemolytic

tests, the stronger the antiserum the less complement was required to produce haemolysis.

Heidelberger, Weil and Treffers (1941) calculated that only 0·3 p.c. of the surface of the erythrocyte need be coated with specific antibody to bring about haemolysis in the presence of complement. The haemolytic systems described here differ from haemolysis as usually described (erythrocytes + specific lysin + complement), in that the antigen is not the erythrocyte itself but the polysaccharide which is attached to the erythrocyte surface; combination of antigen with specific antibody in the presence of complement causes lysis of the erythrocyte. The fact that only small amounts of antibody are required for haemolysis in the presence of excess complement explains the sensitivity of the haemolytic test. Erythrocytes sensitized with weak polysaccharide solutions were lysed in the presence of complement, in higher dilutions of antisera than they were agglutinated. If appropriately applied the haemolytic test offers a method for detecting small amounts of antibody or antigen.

In any study of haemagglutination and haemolysis tests as described here, there arise in the mind of the investigator certain fundamental problems, which have not been explored in this preliminary study—(1) how much polysaccharide is it necessary to adsorb on to the surface of the erythrocyte so that agglutination occurs in a strong antiserum, (2) what is the optimum amount of polysaccharide that must be adsorbed on to the erythrocyte surface in order to reveal the highest possible haemagglutination or haemolytic titre of a specific antiserum, (3) how much of the erythrocyte surface is covered with polysaccharide, (4) how strongly bound is the polysaccharide to the erythrocyte surface and would it be possible to elute the polysaccharide completely and so render the erythrocyte inagglutinable by specific antiserum.

The polysaccharides isolated from *Bact. coli* and *Shigella sonnei* were antigenic, toxic for rabbits (unpublished data) and behaved similarly in most serological tests. Haemagglutination and haemolysis titres of specific antisera were higher than agglutination and precipitation titres and in both cases the vaccine antiserum was more active. Erythrocytes sensitized with *Bact. coli* and *Shigella sonnei* polysaccharides treated with potassium periodate were no longer agglutinated by specific antisera. Hirst (1948) and Goebel, Olitsky and Saenz (1948) have studied the oxidizing effects of the periodate radical on biological materials. The changes induced in *Bact. coli* and *Shigella sonnei* polysaccharides by periodate are difficult to assess but indicate a fundamental difference between the above fractions and those from *Staph. aureus, Staph. albus, Str. pneumoniae* and *C. diphtheriae* as prepared in this study.

The polysaccharides isolated from *C. diphtheriae* and *Staph. aureus* were antigenic and behaved similarly to each other in the haemagglutination, haemolytic and precipitation tests in so much as the polysaccharide antiserum in

each case was more active than the vaccine antiserum. *Staph. aureus* polysaccharide antiserum agglutinated *Staph. aureus* suspensions to a lower titre than the vaccine antiserum, but *C. diphtheriae* suspensions were not agglutinated by *C. diphtheriae* polysaccharide antiserum. *C. diphtheriae* polysaccharide was toxic for rabbits but *Staph. aureus* polysaccharide was not (unpublished data).

SUMMARY.

Haemagglutination and haemolytic tests using sheep erythrocytes, bacterial polysaccharides, complement and specific vaccine and polysaccharide antisera are described.

In the haemagglutination test the concentration of polysaccharide used for sensitization and the sensitizing time affected the observed haemagglutination titre of any specific antiserum. Erythrocytes sensitized with a strong polysaccharide solution for a short time revealed a higher haemagglutination titre of specific antiserum than erythrocytes sensitized with a weaker polysaccharide solution for the same time. Prolonged sensitization of erythrocytes with a strong polysaccharide solution did not reveal a haemagglutination titre higher than that observed after sensitization for one hour. The longer erythrocytes remained in contact with a weak polysaccharide solution, the higher was the observed haemagglutination titre of specific antiserum; after a considerable sensitizing period the same haemagglutination titre as that obtained with erythrocytes sensitized for a short period with a strong polysaccharide solution, was observed.

Addition of complement to the haemagglutination systems caused lysis of the sensitized erythrocytes. The haemolytic test was more sensitive than the haemagglutination test and offers a further method of detecting the presence of small amounts of antibody. Factors mentioned above which affected the haemagglutination titre of a given antiserum also affected the haemolytic titre.

The fact that multiple sensitization of erythrocytes with four polysaccharides did not interfere with specific haemagglutination and haemolytic systems suggested that only a small area of the erythrocyte surface was involved in sensitization.

Agglutination and precipitation titres of specific vaccine and polysaccharide antisera were not as high as the haemagglutination and haemolytic titres.

Acknowledgments. **I am indebted to Mr. N. F. Stanley for helpful advice and to Mr. A. Jennings for chemical examination of the bacterial fractions.**

REFERENCES.

Alexander, M. M., Wright, G. G. and Baldwin, A. C. (1950): J. exp. Med., 91, p. 561.
Fisher, S. and Keogh, E. V. (1950): Nature, 165, p. 248.
Goebel, W. F., Olitsky, P. K. and Saenz, A. C. (1948): J. exp. Med., 87, p. 445.
Hayes, L. and Stanley, N. F. (1950): Austral. J. exp. Biol. (1950), 28, p. 201.
Heidelberger, M., Weil, A. J. and Treffers, H. P. (1941): J. exp. Med., 73, p. 695.
Hewitt, L. F. (1947): Brit. J. exp. Path., 28, p. 338.
Hirst, G. K. (1948): J. exp. Med., 87, p. 301.
Keogh, E. V., North, E. A. and Warburton, M. F. (1947): Nature, 160, p. 63.
Keogh, E. V., North, E. A. and Warburton, M. F. (1948): *Ibid.*, 161, p. 687.
Maculla, E. S. and Cowles, P. (1948): Science, 107, p. 376.
Middlebrook, G. and Dubos, R. J. (1948): J. exp. Med., 88, p. 521.
Platt, A. E. (1941): Austral. J. exp. Biol., 19, p. 57.
Rappaport, F. and Skariton, M. (1949): Amer. J. clin. Path., 19, p. 665.
Warburton, M. F., Keogh, E. V. and Williams, S. W. (1949): Med. J. Austral., 1, p. 135.
Weir, J. M. (1941): Proc. Soc. exp. Biol., 46, p. 47.

THE ARGININE CONTENT OF CERTAIN SEED PROTEINS

by P. HOLMES

(From the Institute of Agriculture, University of Western Australia, Nedlands, W.A.).

(Accepted for publication 2nd October, 1950.)

It has been suggested (Lugg and Weller, 1944) that for the purpose of animal nutrition studies, comprehensive analyses of pasture proteins might be confined to a few species belonging to the most important families of pasture plants. In the case of special proteins such as the seed proteins, however, no such relatively uniform amino acid composition exists. Partial amino acid analyses of seed proteins must then, of necessity, be made with the individual types of seeds themselves for use in animal nutrition studies, as even closely related seed proteins exhibit very varied amino acid compositions.

Arginine contents of certain seed proteins have been determined, the analyses being made by Vickery's (1940) modified flavianic acid method and based on "whole" protein preparations of the seeds. The seeds selected for analysis are those commonly used as a protein source for sheep during the summer months. It is intended to extend the partial amino acid analyses of these seeds, particularly for those amino acids listed as being nutritionally essential in the diet (Rose, 1938) and, in a subsequent paper, to discuss the nutritional significance of the results in relation to the wool growth of Merino sheep.

EXPERIMENTAL.

Preparation of materials.

The seeds selected for analysis were Lupin (*Lupinus pilosus* W.A. blue); Pea (*Pisum sativum* var. White Brunswick); Vetch (*Vicia sativum*); Subterranean Clover (*Trifolium subterraneum*) and a commercial product of Linseed (*Linum usitatissimum*).

TABLE 1.

Details of "whole" protein preparation.

Seed Material	Fresh wt. (gm.)	P.c. moisture	Dry wt. (gm.)	P.c. N	Total N (mg.)	Protein N (mg.)	Dry wt. protein prep. (gm.)	P.c. N protein prep.
Sub-clover	29·91	5·48	28·36	5·64	1,599	1,224	17·21	7·11
Linseed	30·33	6·54	28·35	6·30	1,786	1,477	22·61	6·53
Vetch	32·97	9·52	29·83	4·52	1,348	1,147	26·25	4·37
Pea	31·93	11·1	28·38	4·55	1,291	1,164	23·96	4·44
Lupin	30·73	10·2	27·60	5·47	1,510	1,371	23·96	5·72

"Whole" protein preparations were prepared from the ground seeds by the method of Lugg (1939) with Lugg and Weller's (1944) modification, the process involving denaturation and precipitation of the protein and removal of non-protein nitrogenous compounds and some others, while retaining a certain quantity of carbohydrate material.

Data concerning the fresh material and the protein preparations made from it are shown in Table 1.

Analytical work.

Nitrogen determinations were carried out using the micro-Kjeldahl method of Willits, Coe and Ogg (1949) with HgO as the catalyst.

For the estimation of arginine the method of Kossel and Gross (1924) with Vickery's (1940) modification was used in which arginine was first precipitated from the acidic hydrolysate as the diflavianate and subsequently converted to and weighed as arginine monoflavianate. Close replicates were obtained for each sample analysed.

RESULTS.

The results of the analyses are shown in Table 2, the arginine contents of the seeds being expressed as a percentage arginine–N of protein N.

TABLE 2.

Arginine contents of "whole" protein preparations.

Seed material	Dry weight seed (gm.)	Dry weight protein (gm.)	P.c. N	P.c. arginine N of protein N
Sub-clover	28·36	17·21	7·11	12·3
Linseed	28·35	22·61	6·53	13·3
Vetch	29·83	26·25	4·37	17·6
Pea	28·38	23·96	4·44	12·9
Lupin	27·60	23·96	5·72	15·9

The arginine values are uncorrected for losses sustained during hydrolysis of the protein in the presence of carbohydrate material which makes up the bulk of the impurity in the preparation. That serious losses of arginine do occur in the presence of carbohydrates seems undoubted as shown by Tristram (1939) who observed that the yield of arginine decreased as the quantity of carbohydrate in the preparation increased. Tristram proposed an empirical correction based on the nitrogen content of the preparation. Roche and Blanc-Jean (1940) confirmed the findings of Hunter and Dauphinee (1929) that the number of arginine residues decreases with increasing time of hydrolysis. Most attempts to evaluate hydrolytic losses have involved addition of the amino acid both before and after hydrolysis; the apparent destruction then being determined by the difference in the analytical result obtained. Such a correction involves the assumption that the free amino acid added reacts during hydrolysis in exactly the same way as its residue in the peptide linkage, a fact known to be untrue (see Shemin, 1946; Roxas, 1916). Lugg and Weller (1948a) found that losses in the absence of carbohydrate were zero and that the losses due to the presence of such material are proportional to the amount of it, if the other variables are kept constant. Calculation of the amount of carbohydrate impurity was made from the known nitrogen content of the hypothetically "pure" protein and corrections

to the analytical figures were made accordingly. It is felt, however, that a correction of this type entails serious uncertainties, even for the leaf-protein preparations of Lugg and Weller (1948a). Furthermore, in view of the low nitrogen contents of the seed protein preparations and the fact that it is difficult to judge what the nitrogen contents of the hypothetically "pure" proteins might be, it has been considered inadvisable to attempt to correct the values. It must be realized, therefore, that these values are almost certain to be somewhat low.

DISCUSSION.

From Table 1 the sub-clover sample is seen to have contained a large amount of extractable non-protein material compared with the remaining samples. This is in conformity with the values reported by Lugg and Clowes (1945) who stated p.c. total N as 5·80 and p.c. N in the protein preparation as 7·69 for the sample of seed examined.

Data for the vetch and pea preparations indicate that the proportions of non-protein nitrogenous substances to the other extractable substances is large compared with some other seed preparations (Lugg and Weller, 1944; Johanson and Lugg, 1946). The percentage nitrogen in the "whole" protein preparation is lower than in the unextracted seeds.

The vetch sample which has the lowest protein content, a value of 4·37 for the p.c. N in the protein preparation, has the highest value (17·3) for the percentage arginine-N of protein N, indicating a high proportion of arginine in the relatively small amount of protein in the seed. Quite the reverse is the case with the sub-clover sample which has a high protein N content (7·11) but has the lowest value (12·3) for the percentage arginine-N of protein N.

Comparative results from the literature are meagre. Block and Bolling (1945) report a value for linseed of 13·8 for the percentage arginine-N of protein N. No reliance can be placed on this figure as the percentage nitrogen content is not given. Block and Bolling also report a value of 11·1 p.c. for the globulin of pea (vicilin) and 17·8 p.c. for the albumin (legumelin). The value determined here for the whole protein of pea seed is 12·9, a value lying between those of legumelin and vicilin and thus of interest as to the variation in the proportion of proteins in the whole protein of the seed.

It is well known that change of environmental conditions will modify the content of non-protein constituents as well as that of the proteins. A survey of the literature, however, elicits very little pertaining to the all important question of the variation of amino acid composition with changes in environment. Lugg and Weller (1948b) found that the protein composition of young leaf tissue differed markedly from that of the whole protein in older tissue. The authors were unable to say whether this variability was brought about by a change in composition of the individual proteins or whether the proportions of the individual proteins making up the whole protein had undergone

change. The tacit assumption seems to be that such compositions of individual proteins are constant. For this reason the analytical results are recorded as a percentage of the protein nitrogen content, from which figure, together with the protein nitrogen content, a simple calculation will give the quantity of seed protein arginine.

SUMMARY.

The arginine contents in protein preparations of the seeds of lupin (*Lupinus pilosus*), vetch (*Vicia sativum*), linseed (*Linum usitatissimum*), pea (*Pisum sativum*) and subterranean clover (*Trifolium subterraneum*) are reported; the values expressed as a percentage arginine-N of protein N are 15·9, 17·6, 13·3, 12·9, 12·3 respectively.

It has been considered inadvisable to attempt to correct these values for analytical losses which are believed to have occurred but which are of uncertain magnitude in the present state of our knowledge. It is apparent that these seed proteins cannot be regarded as deficient in arginine from the point of view of the nutritional requirements of animals.

Acknowledgments. Grateful acknowledgment is made to the Wool Research Trust Funds for financial assistance and to Professors E. J. Underwood and J. W. H. Lugg for their helpful criticism.

REFERENCES

Block, R. J. and Bolling, D. (1945): "The Amino Acid Compositions of Proteins and Foods." Charles C. Thomas, Springfield, Ill., U.S.A.

Hunter, A. and Dauphinee, G. S. (1929): J. biol. Chem., 85, p. 627.

Johanson, R. and Lugg, J. W. H. (1946): Austral. J. exp. Biol., 24, p. 321.

Kossel, A. and Gross, R. E. (1924): Z. physiol. Chem., 135, p. 166.

Lugg, J. W. H. (1939): Biochem. J., 33, p. 110.

Lugg, J. W. H. and Weller, R. A. (1944): Austral. J. exp. Biol., 22, p. 149.

Lugg, J. W. H. and Clowes, G. J. (1945): *Ibid.*, 23, p. 75.

Lugg, J. W. H. and Weller, R. A. (1948a): Biochem. J., 42, p. 408.

Lugg, J. W. H. and Weller, R. A. (1948b): *Ibid.*, 42, p. 412.

Roche, J. and Blanc-Jean, G. (1940): Comp. rend. Acad. Sciences, 210, p. 681; from Chem. Abs. (1940), 34, p. 5,102.

Rose, W. C. (1938): Physiol. Rev., 18, p. 109.

Roxas, M. L. (1916): Biochem. J., 27, p. 71.

Shemin, D. (1946): Ann. N.Y. Acad. Sci. (from Biochem. Rev., 1946).

Tristram, G. R. (1939): Biochem. J., 33, p. 1,271.

Vickery, H. B. (1940): J. biol. Chem., 132, p. 325.

Willits, C. O., Coe, M. R. and Ogg, C. L. (1949): J. Assoc. off agric. Chem., 32, p. 118.

THE PRESENCE OF "BOUND" HISTAMINE FOLLOWING THE INJECTION OF HISTAMINE ACID-PHOSPHATE INTO THE PERFUSED LIVER

by E. R. TRETHEWIE AND FAY M. GAFFNEY[1]

(From the Institute of Medical and Veterinary Science, Adelaide and the Department of Experimental Medicine, University of Adelaide).

(Accepted for publication 2nd August, 1950.)

Trethewie and Day (1949) using the method described by Anrep, Ayadie, Barsoum, Smith and Talaat (1944) for the detection of conjugated histamine in the urine, found that a "bound" form of histamine was liberated into the perfusates of blood-perfused livers of dogs following the injection of venom of the Australian black snake (*Pseudechis porphyriacus*) into the portal vein. In the present experiments, an attempt has been made to demonstrate the presence of conjugated histamine in the perfusates of dogs' livers after the injection of histamine acid-phosphate into the portal vein. Also we have endeavoured to exclude the possibility that the "bound" histamine is formed in the blood. While this work was being completed Millican, Rosenthal and Tabor (1949) produced evidence for the formation of conjugated histamine by co-enzyme-A, acetone liver powder and histamine.

METHOD.

Tyrode Perfusion.

In two instances, dogs were anaesthetized with Pentothal given intravenously (30 mg./kg.) and the liver of each was perfused intraportally with Tyrode at 36° C. by the method described by Feldberg and Kellaway (1937). The bulk of the organ was tied off and excised. The right lateral lobe weighing 72–145 gm. was used for perfusion. Extracts were made of the left lobes excised and of the right lobes after perfusion. Both were boiled at once to destroy inactivating enzymes. The rate of perfusion of the lobe through the portal vein was 0·02–0·03 ml./gm./min. 3 mg. of histamine acid phosphate (0·03–0·05 mg./gm. liver) were injected through the cannula into the portal vein and the flow was interrupted for one minute. S_1 perfusate was collected before and S_2–S_8 after 0–5, 5–15, 15–30, 30–60, 60–90, 90–120, 120–150 minutes' perfusion after the injection of histamine acid phosphate in one experiment and in the other experiment S_2–S_7 after 0–4, 4–21, 21–34, 34–49, 49–65 and 65–94 minutes' perfusion. The samples were boiled immediately after collection. The liver was drained for 30 minutes and the sample boiled.

[1] This work was aided by a grant from the National Health and Medical Research Council.

Blood Perfusion.

Dogs were anaesthetized with intravenous Pentothal (30–34 mg./kg.). Blood (150–235 ml.) was withdrawn from the iliac artery into a cylinder containing a solution of Boots heparin (making 0·12 mg./ml.), mixed well and then diluted with an equal volume of Tyrode. When the lobe was perfused free of obvious blood (as above), the Tyrode was replaced by diluted heparinized blood. A perfusion was carried out then as before. Samples were immediately boiled (S_1–S_9 in one instance, S_1–S_7 in three instances and S_1–S_5 in three instances). The times corresponded to those of the first Tyrode experiment above. S_9 was collected at 150–180 minutes. The time of perfusion was shortened as later samples contained negligible amounts of histamine. The perfusion rate was 0·001–0·03 ml./gm. liver/min. In four experiments a portion of each sample was centrifuged at 4,000 r.p.m. for 30 minutes and the plasma boiled thereafter.

Division Experiments.

The perfusion was similar to the blood perfusion, except that the perfusate was divided into approximately two equal volumes. Each drop of out-flowing perfusate was divided into almost equal portions by allowing it to fall on to a serrated knife edge set on an angled sheet of celluloid, as described by Kellaway and Trethewie (1940). One part was collected at room temperature (A), the volume measured, and a portion of this was boiled after collection and the remainder was centrifuged and the plasma boiled. The other part was immediately heated, making measurement of volume unsatisfactory as it was collected at 95° C. (B). Enzymes therefore could not act in (B) during the time of collection. In one experiment heparinized blood diluted with an equal volume of Tyrode was used. As very little fluid could be obtained from (B) in the other experiments, blood diluted 1/4 with Tyrode was used for the perfusions. In two experiments (B) was collected at 4° C. (volume being measured) and immediately boiled after collection.

Adsorption Experiments.

Livers were perfused with diluted heparinized blood as in the blood perfusions. The S_2–S_5 samples were pooled and centrifuged and the plasma treated by the procedure used by Anrep, Ayadie, Barsoum, Smith and Talaat (1944) for the estimation of conjugated histamine in urine. The final residue was extracted as described in their paper by a method similar to that given by Barsoum and Gaddum.

Histamine.

Histamine was assayed as histamine acid-phosphate on the isolated jejunum of the guinea-pig suspended in a bath of Tyrode. Tests were carried out also after the addition of Neoantergan (N-dimethyl amino ethyl N-methoxy benzo α-amino pyridine) to the bath.

EXPERIMENTAL.

Tyrode Perfusion (Liver): Injection of Histamine.

Two experiments were carried out using Tyrode for the perfusion. 3 mg. of histamine acid phosphate were injected in both instances. 55 p.c. of the amount of histamine injected was recovered in the perfusates and drainage fluids in one experiment and 88 p.c. in the other (average of 72 p.c.).

Blood Perfusion (Liver): Injection of Histamine.

In four instances heparinized blood diluted 1:1 with Tyrode was used and the flow interrupted for 1 minute while 3 mg. histamine were injected into the portal vein. An average of 23 p.c. histamine was recovered in the perfusates, i.e. less than half the amount obtained in

the Tyrode perfusates (Table 1). There are three possibilities to account for this. It is possible that the liver "mops" up the histamine so that it does not appear in the perfusates. Because a blood perfusion experiment is more nearly physiological some histamine may be converted into an inactive substance in the liver or the blood, and may appear in the perfusates undetected in a "bound" form. It is also possible that enzymes in the blood may destroy some histamine during the time of perfusion.

TABLE 1.

Recovery of 3 mg. histamine injected into the portal vein.

Tyrode perfusion			Blood perfusion		
Dog No.	P.c. recovery	Time	Dog No.	P.c. recovery	Time
1	55	94 mins.	3	27	165 mins.
2	88	150 mins.	4	18	150 mins.
			5	21	120 mins.
			6	27	120 mins.
Av.	72		Av.	23	

TABLE 2.

P.c. recovery of histamine in whole blood and plasma when 10 μg. histamine injected during 60 minutes' perfusion (including drainage).

Dog No.	Whole blood (p.c. recovery)	Plasma (p.c. recovery)
8	36	43
9	32	60
11	15	56
12	48	46
13	15	15

A comparison of the amount of histamine as assayed on the gut from whole blood and plasma was made (Table 2).

In experiment 8, the whole blood was boiled immediately after the collection of each sample whereas the plasma was boiled after all the samples were collected and the plasmas centrifuged for ½ hour. In experiments 9, 11, 12 and 13 the whole blood and plasma were boiled at the same time. The smaller amount in the whole blood (generally found) may be ascribable to inhibitor substances present in the whole blood.

TABLE 3.

P.c. recovery of histamine in whole blood inactivated immediately and afterwards (drainage excluded).

Dog No.	Whole blood boiled or frozen at once (P.c. recovery)	Whole blood boiled afterwards (P.c. recovery)
11	30	15
12	26	38
13*	10	12
14*	38	34

* 4° C. used for inhibiting enzyme activity instead of 95° C.

The destruction of histamine by enzymes in the blood usually played only a minor part in the incomplete recovery of histamine in the liver perfusates. On the average 26 p.c. recovery was obtained when the whole blood was boiled or chilled as it collected and 25 p.c. when it was boiled afterwards (Table 3). This difference cannot explain the difference between the Tyrode and blood perfusions. Blood in the liver may be more active or the liver itself may be responsible in a blood perfusion. Further, in experiments 12 and 13 there is an "apparent" increase in the histamine content in the whole blood boiled afterwards (Table 3). In No. 11, the figure for the whole blood boiled after does not include the histamine in a large volume sample which only contained a trace of histamine for which we could not give a figure but in which a small concentration is not negligible.

The destruction of histamine by enzymes in the blood is demonstrated by adding histamine acid phosphate to whole blood (diluted 1: 1 with Tyrode) and incubating at 36° C. for one and four hours and testing. In experiment 13, when 1/1·1 mill. histamine was present in whole blood, 27 p.c. was lost during one hour's and 35 p.c. during four hours' incubation. When 1/17 mill. histamine was present in whole blood, no detectable amount was lost during one hour and 32 p.c. during four hours' incubation (Table 4).

TABLE 4.

P.c. loss of histamine during incubation of diluted heparinized blood at 36° C.

Dog No.	Amount of histamine (μg./ml.)	Whole blood	
		P.c. loss (60 mins.)	P.c. loss (240 mins.)
10	1·0	40	67
11	1·0	28	47
	0·1	slight	17
12	1·0	8	35
13	0·99	27	35
	0·06	nil	32

Conjugation of Histamine by the Blood Perfused Liver.

Ten experiments were carried out on extract samples by the method of Anrep and his co-workers. In all of them, a greater amount of histamine was detected in the hydrolysed eluate than in the unhydrolysed eluate when assayed on the jejunum of the guinea-pig (Table 5). In experiment 21, no histamine was detected in S_{2-5}, pooled plasma boiled. A "slow-reacting substance" caused a contraction of the gut with a latency of 5 seconds. In Fig. 1a are shown the responses to eluate obtained from this experiment. 0·4 ml. hydrolysed eluate (concentrated 3/2) contained a concentration of histamine 1/13 mill., so that the hydrolysed eluate contained 1/20 mill. (H.E. Fig. 1a). 0·4 ml. unhydrolysed eluate (concentrated 3/2) contained no detectable histamine when assayed on the gut (U.E. Fig. 1a). The unhydrolysed eluate represents the amount of free histamine (assuming the absence of inhibitor) and the hydrolysed eluate the total amount of histamine after hydrolysis. Therefore 1/20 mill. represented the concentration of histamine in a "bound" form which could not be assayed on the gut until it was hydrolysed. No histamine was detected in the filtrate. The histamine appeared to be adsorbed completely on to the charcoal. 0·6 ml. unhydrolysed filtrate (concentrated 3/2) gave a contraction after 6 seconds (U.F. Fig. 1b) and 0·6 ml. hydrolysed filtrate (concentrated 3/2) gave an immediate contraction, but on washing the bath, the gut relaxed slowly and the sensitivity to histamine was increased (H.F. Fig. 1b). The S.R.S. did not appear to be adsorbed on to the charcoal and it passed into the filtrate. 0·5 μg. Neo-antergan was added to the bath and left in contact with the gut for 30 seconds (N.A. Fig. 1c). Now 0·1 μg. histamine did not cause the gut to

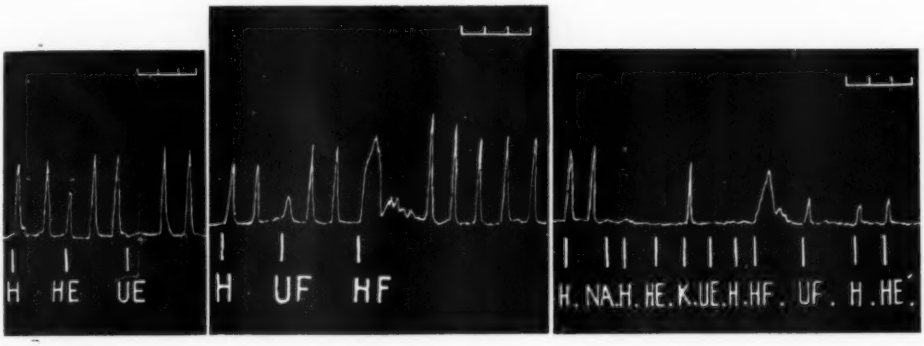

Fig. 1a. **Fig. 1b.** **Fig. 1c.**

Fig. 1a. Responses of the isolated jejunum of the guinea-pig to the eluate fractions in Exp. 21. At H.E. 0·4 ml. hydrolysed eluate (3/2 conc.) contained a histamine concentration of 1/13 mill. At U.E. 0·4 ml. unhydrolysed eluate (3/2 conc.) contained no detectable histamine. H. equivalent to 0·1 μg. histamine. Unlettered contractions to 0·1 μg. histamine.

Fig. 1b. Responses of the isolated jejunum of the guinea-pig to the filtrate fractions in Exp. 21. At U.F. 0·6 ml. unhydrolysed filtrate (3/2 conc.) gave a contraction after 6 seconds. At H.F. 0·6 ml. hydrolysed filtrate (3/2 conc.) gave an immediate contraction after which the sensitivity of the gut to histamine was increased. H equivalent to 0·1 μg. histamine. Unlettered contractions to 0·1 μg. histamine.

Fig. 1c. Response of the isolated jejunum of the guinea-pig after the addition of neo-antergan to the bath. At N.A. 0·5 μg. neo-antergan injected. At H. 0·1 μg. histamine gave no reaction. At U.E. 0·4 ml. unhydrolysed eluate produced no contraction. At H.E. 0·4 ml. hydrolysed eluate did not cause gut to contract. At U.F. (unhydrolysed filtrate) and H.F. (hydrolysed filtrate) 0·6 ml. of each caused the gut to contract. At K, 0·4 mg. K injected. H equivalent to 0·1 μg. histamine. At H.E'. 0·8 ml. hydrolysed eluate caused a contraction. Unlettered contractions equivalent to 0·1 μg. histamine.

TABLE 5.

Comparison of the amount of histamine in the unhydrolysed (U.E.) and hydrolysed eluates (H.E.).

Dog No.	S_2–S_5 boiled plasma (μg.)	U.E. (μg.)	H.E. (μg.)	Conjugated histamine (μg.)
15	0·89	0·89	1·3	0·41
16	Nil	Nil	1·5	1·50
17	Nil	1·0	5	4·00
18	1·0	0·83	2·0	1·17
19	1·2	Trace	4·0	4·00
20	3·7	3·7	9·1	5·40
21	Nil	Nil	3·0	3·00
22	1·5	1·5	9·2	7·70
25	0·067	Nil	0·033	0·03
26	0·14	0·033	0·059	0·03

contract (H. Fig. 1c). No contraction was produced by 0·4 ml. unhydrolysed eluate (U.E. Fig. 1c) or 0·4 ml. hydrolysed eluate (H.E. Fig. 1c). 0·6 ml. unhydrolysed filtrate (U.F. Fig. 1c) and 0·6 ml. hydrolysed filtrate (H.F. Fig. 1c) caused the gut to contract when it did not react to 0·1 μg. histamine. A sharp contraction with a rapid relaxation was obtained with 0·4 mg. K (K. Fig. 1c). When the gut began to react to histamine so did it react to 0·8 ml. hydrolysed eluate (H.E'. Fig. 1c). It would appear that the contraction produced by the hydrolysed eluate was due to histamine as it was abolished by Neo-antergan and as the gut recovered, it contracted to both the histamine acid phosphate and hydrolysed eluate. The contractions produced by the filtrate portions were not abolished by the Neo-antergan.

TABLE 6.

P.c. of undetected histamine in the perfusate, accounted for by conjugated histamine.

Dog No.	Amt. conj. hist. (H.E.—U.E.) μg.	Amt. hist. undetected in perf. (10 μg. — content S_2–S_5 boiled plasma)	P.c. of undetected hist. as conj. hist.
15	0·41	9·11	5
16	1·50	10·00	15
17	4·00	10·00	40
18	1·17	9·00	13
19	4·00	8·80	46
20	5·40	6·30	85
21	3·00	10·00	30
22	7·70	8·50	90
25	0·03	9·93	0·3
26	0·03	9·86	0·3

In experiments 17 and 18 no increase in the titres of histamine by the unhydrolysed and hydrolysed parts of the eluate was detected after adsorption with alumina (B.D.H.). The relaxation below the base line sometimes occurring on washing the bath was not abolished after the adsorption. In experiments 19 and 22, the titres of histamine were the same before and after the adsorption with alumina. In experiment 18, the hydrolysed eluate before adsorption contained 1/27 mill. and the unhydrolysed eluate 1/60 mill. After adsorption with aluminium oxide, the unhydrolysed eluate had a concentration of 1/60 mill. and the hydrolysed eluate 1/23 mill. The relaxing substance present in both fractions caused the gut to relax below the base line when the bath was washed with Tyrode and it did not appear to be adsorbed on to the alumina. In Fig. 2a, at H.E. 0·6 ml. hydrolysed eluate (concentrated 3/2) is equivalent to 0·034 μg. histamine and 0·6 ml. unhydrolysed eluate (concentrated 3/2, U.E.) to 0·015 μg. histamine before adsorption. In Fig. 2b, 0·6 ml. hydrolysed eluate (3/2 concentration, H.E'.) is equivalent to 0·04 μg. histamine and 0·6 ml. unhydrolysed eluate (concentrated 3/2, U.E'.) to 0·015 μg. histamine after adsorption. It would appear therefore that inhibitors to histamine were not present in the eluate. To confirm these results, in experiment 19, 0·1 ml. histamine (1/200,000) was added to 0·9 ml. of unhydrolysed eluate and to 0·9 ml. of hydrolysed eluate. If no histamine inhibitors were present 0·1 ml. of this mixture (unhydrolysed eluate) (H' Fig. 3) should be equivalent to 0·05 μg. histamine (H Fig. 3). If anything in Fig. 3, it (H') is a little greater. Also 0·1 ml. of this mixture with the hydrolysed eluate (H") is equal to 0·05 μg. histamine in its contraction. In one experiment (No. 26) a difference in the contraction was noted after adsorption with alumina. The unhydrolysed eluate contained 1/80 mill. concentration of histamine before and 1/30 mill. after adsorption with Al_2O_3 and the hydrolysed eluate contained 1/24 mill. concentration of histamine before and 1/17 mill. after

adsorption. These findings are shown in Fig. 4 where 0·6 ml. unhydrolysed eluate (2 × concentration, U.E.) is equivalent to 0·015 μg. histamine and 0·6 ml. unhydrolysed eluate after adsorption (2 × concentration, U.E'.) is equivalent to 0·04 μg. histamine. 0·6 ml. hydrolysed eluate (2 × concentration, H.E.) is equivalent to 0·05 μg. and 0·6 ml. hydrolysed eluate (2 × concentration, H.E'.) is equivalent to 0·07 μg. histamine after adsorption. It would appear that the presence of histamine inhibitors in the unhydrolysed eluate did not account for the difference in the contractions produced by the unhydrolysed and hydrolysed fractions of the eluate. In the instance where it was detected it was only of the order of inhibiting 1/50 mill. histamine.

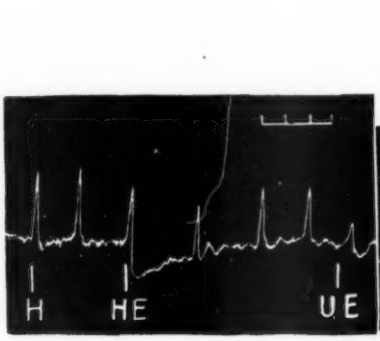

Fig. 2a.

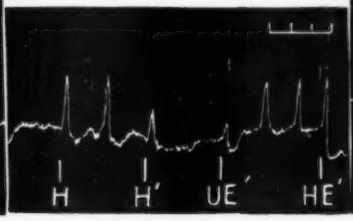

Fig. 2b.

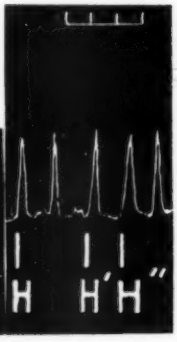

Fig. 3.

Fig. 2a. Responses of the isolated jejunum of the guinea-pig to the eluate fractions of experiment 18, before adsorption on to alumina. At H.E. 0·6 ml. hydrolysed eluate (3/2 conc.) is equivalent to 0·034 μg. histamine. At U.E. 0·6 ml. unhydrolysed eluate (3/2 conc.) is equivalent to 0·015 μg. H equivalent to 0·04 μg. and unlettered contractions to 0·04 μg. histamine.

Fig. 2b. Responses of the isolated jejunum of the guinea-pig to the eluate fractions of experiment 18 after adsorption on to alumina. At H.E'. 0·6 ml. hydrolysed eluate (3/2 conc.) is equivalent to 0·4 μg. histamine. At U.E'. 0·6 ml. unhydrolysed eluate (3/2 conc.) equivalent to 0·015 μg. histamine. H. is equivalent to 0·04 μg. H' is equivalent to 0·02 μg., unlettered contractions to 0·04 μg. histamine.

Fig. 3. Responses of the isolated jejunum of the guinea-pig to eluate fractions to which histamine (1/2 million final concentration) is added in experiment 20. At H' 0·1 ml. of unhydrolysed eluate + histamine is approximately equivalent to 0·05 μg. At H" 0·1 ml. hydrolysed eluate + histamine is equivalent to 0·05 μg. H is equivalent to 0·05 μg. histamine, unlettered contractions to 0·05 μg. histamine.

Control Experiments.

The blood collected prior to the injection of histamine did not contain conjugated histamine, e.g. in experiment 22, S_1 was collected before the injection of histamine and S_2–S_5 (pooled) after the injection. Both samples of plasma were subjected to the treatment for the detection of conjugated histamine. When assayed on the gut, S_1 boiled plasma contained S.R.S. only and S_2–S_5 contained histamine 1/30 mill. Assay of the eluates is shown in Fig. 5. 0·6 ml. unhydrolysed eluate from S_1 contained no histamine (U.E. Fig. 5a), nor did the hydrolysed eluate (H.E. Fig. 5a). A slow contraction with a latency of 9 seconds was caused

by both fractions. The unhydrolysed eluate from S_2–S_5 boiled plasma (U.E. Fig. 5b) contained 1/30 mill. concentration of histamine and the hydrolysed eluate from S_2–S_5 boiled plasma (H.E. Fig. 5b) contained histamine 1/5 mill.

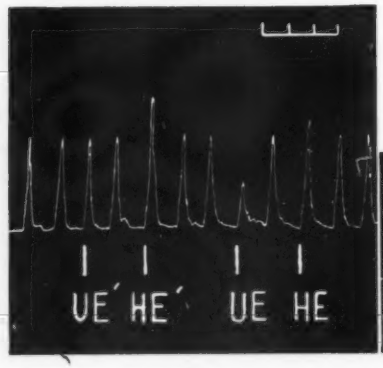

Fig. 4.

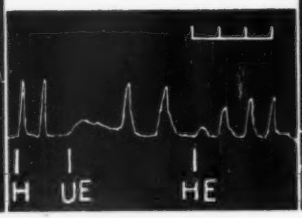

Fig. 5a.

Fig. 5b.

Fig. 4. Responses of the isolated jejunum of the guinea-pig to the eluate fractions before and after adsorption with alumina in Exp. 26. At U.E. 0·6 ml. unhydrolysed eluate (2 × conc.) is equivalent to 0·015 μg. histamine. At U.E'., 0·6 ml. unhydrolysed eluate (2 × conc.) after adsorption is equivalent to 0·04 μg. At H.E. 0·6 ml. hydrolysed eluate (2 × conc.) is equivalent to 0·05 μg. histamine. At H.E'. 0·6 ml. hydrolysed eluate (2 × conc.) after adsorption is equivalent to 0·07 μg. histamine. Unlettered contractions to 0·04 μg. histamine.

Fig. 5a. Responses of the isolated jejunum of the guinea-pig to S_1 eluate fractions in control experiment 22. At U.E. 0·6 ml. unhydrolysed eluate contained no histamine. At H.E. 0·6 ml. hydrolysed eluate did not cause an immediate contraction. A slow contraction with a latency of 9 seconds was caused by both U.E. and H.E. H is equivalent to 0·1 μg. histamine. Unlettered contractions to 0·1 μg. histamine.

Fig. 5b. Responses of the isolated jejunum of the guinea-pig to S_2–S_5 eluate fractions in control experiment 22. At U.E. 0·6 ml. unhydrolysed eluate (equivalent to 0·02 μg.). At H.E. 0·6 ml. hydrolysed eluate is equivalent to 0·12 μg. histamine. H is equivalent to 0·1 μg. histamine, unlettered contractions to 0·1 μg. histamine.

In experiment 23, blood was withdrawn from the left iliac artery and collected in a cylinder containing heparin to make a concentration of 0·12 mg./ml. A blood perfusion was set up and the blood collected from a cannula directly on to a paraffin plate into a beaker, i.e. in the same way as for a blood perfusion of the liver, without the liver. Also blood was incubated at 36° C. for 60 minutes with 1/10 million concentration of histamine. The unhydrolysed eluate of the normal blood diluted 1:1 with Tyrode contained no detectable histamine when assayed on the gut. Nor did the hydrolysed eluate contain histamine. The unhydrolysed parts of the filtrate caused the gut to contract slowly and to relax slowly before washing the bath with Tyrode. The contraction was therefore not characteristic of a histamine contraction and was not abolished by Neo-antergan. 0–4 ml. unhydrolysed eluate (7/4 concentration U.E. Fig. 6a) did not cause the gut to contract and 0·4 ml. hydrolysed eluate (7/4 concentration H.E. Fig. 6a) caused a slow short contraction not typical of histamine. Fig. 6b shows the reactions of the gut to the hydrolysed and unhydrolysed fractions of the eluate, obtained from the blood incubated with 1/10 mill. histamine. Both the hydrolysed and unhydrolysed eluates contained 1/13 million concentration of histamine. 0·6 ml. hydrolysed eluate (7/4 con-

centration H', Fig. 6b) is equivalent to less than 0·1 μg. histamine (actually equal to 0·08 μg. histamine) and 0·6 ml. unhydrolysed eluate also is equivalent to less than 0·1 μg. histamine (actually equal to 0·08 μg. histamine) (7/4 concentration H", Fig. 6b).

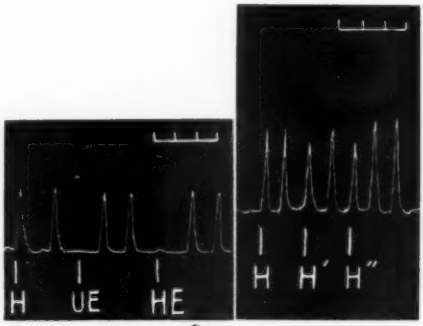

Fig. 6a. Fig. 6b.

Fig. 6a. Responses of the isolated jejunum of the guinea-pig to the eluate fractions in control experiment 23. At U.E. 0·4 ml. unhydrolysed eluate (7/4 conc.) caused no reaction. At H.E. 0·4 ml. hydrolysed eluate (7/4 conc.) caused slow contraction. H is equivalent to 0·1 μg. histamine, unlettered contractions to 0·1 μg. histamine.

Fig. 6b. Responses of the isolated jejunum of the guinea-pig to the eluate fractions in control experiment 23, when histamine is added. At H', 0·6 ml. hydrolysed eluate (7/4 conc.) is equivalent to 0·08 μg. At H", 0·6 ml. unhydrolysed eluate (7/4 conc.) is equivalent to 0·08 μg. H is equivalent to 0·1 μg. histamine, unlettered contractions to 0·1 μg. histamine.

From the control experiments one concludes that the blood does not contain conjugated histamine normally, nor is it able to conjugate any histamine that is present in the blood. Also conjugated histamine is not released into the liver perfusates until the histamine is injected into the portal vein. Whether the conjugation takes place in the blood in contact with the liver cell or on the cell surface or within the cell is not determined in these experiments.

DISCUSSION.

It has been shown in these experiments that the amount of histamine (assayed by the guinea-pig jejunum method) in the liver perfusates, following the injection of histamine into the portal vein, is considerably less in the blood perfusion than in the Tyrode perfusion experiments. There are three possible explanations for the decreased amount of histamine in the blood perfusions. Firstly, enzymes present in the blood or liver may destroy some histamine. It would appear from the experiments in which histamine was incubated with diluted heparinized blood that this activity does not explain wholly the difference between the amounts of histamine obtained in Tyrode and blood perfusions. Secondly, the liver may retain the histamine, which would appear later in the perfusate. It was found that after one hour's perfusion the amounts of histamine found in the later samples were very small. Finally, the histamine may be converted into an inactive form and pass into the perfusates undetected by the gut assay. Trethewie and Day (1949) in experiments with black snake venom, found a small amount of "bound" histamine in the perfusates from blood perfused livers. Anrep, Ayadie, Barsoum, Smith and Talaat (1944) found that a small amount of ingested histamine was detected conjugated in the urine of dogs. Tabor and Mosettig showed that this histamine is in

acetylated form. Anrep *et al.* suggested that the conjugation of histamine may occur in the liver and (or) the intestinal mucosa, as histamine injected intravenously was immediately excreted in the urine in a free form; the conjugated form was absent. From the experiments we have performed it would appear that the liver plays a part in the conjugation of histamine. In all the experiments, a greater amount of histamine was detected in the hydrolysed portion of the eluate. After adsorption of the eluate with alumina, the hydrolysed fraction still contained more histamine than the unhydrolysed fraction and was practically unaltered in concentration, the presence of alumina-adsorbable inhibitors being unusual. The histamine apparently is completely adsorbed on to the charcoal and the S.R.S. remains in the filtrate fractions. After the addition of Neo-antergan to the bath there occurred no reaction to a certain dose of histamine and of the eluate fractions, when a brisk response of the gut occurred with a dose of potassium of the same order. When the response of histamine was regained the gut responded to the eluate portions. In Table 6 a certain amount of undetected histamine in the S_2–S_5 boiled plasma, by gut assay as histamine acid phosphate, may be represented by the conjugated form of histamine which passes into the perfusate undetected. Histamine when mixed with blood alone and incubated was not conjugated in our experiments.

It would seem that the liver and perhaps other organs of the body, inactivate free histamine. It is possible that histamine is inactivated by this mechanism in the body when liberated being a more efficient process than the very slow inactivation with histaminase.

SUMMARY.

Conjugated histamine is liberated into the blood perfusates when histamine is injected into the portal vein. The evidence that this is histamine following hydrolysis is that the contractions produced by the eluate parts are abolished by Neo-antergan and are regained as the histamine contractions are restored.

The histamine is adsorbed on to charcoal and adsorption with alumina of the unhydrolysed and hydrolysed portions of the eluate does not usually reveal the presence of histamine inhibitors.

REFERENCES.

Anrep, G. V., Ayadie, M. S., Barsoum, G. S., Smith, J. R. and Talaat, M. M. (1944): J. Physiol., 103, p. 155.

Feldberg, W. and Kellaway, C. H. (1937): J. Physiol., 90, p. 257.

Kellaway, C. H. and Trethewie, E. R. (1940): Austral. J. exp. Biol., 18, p. 63.

Millican, R. C., Rosenthal, M. C. and Tabor, H. (1949): J. Pharm. and exp. Therap., 97, p. 4.

Tabor, H. and Mosettig, E.: Unpublished work quoted by Millican, Rosenthal and Tabor (1949).

Trethewie, E. R. and Day, Allan J. (1949): *Ibid.*, 27, p. 385.